

Justice unevenly spread in Paris

The second version of the contaminated-blood trial in the French courts leaves the suspicion that those sent to jail are a subset of those who might have been so dealt with.

How should a researcher strike a balance between loyalty to his or her employer and loyalty to the stated goals and inferred responsibilities of the same organization? That is, of course, a particular version of an old question: does a person's duty to his employer require compliance with orders, even when these are likely to offend and even damage the public interest, or the interests of individuals who may be involved? Even in the military field, where the maxim 'orders are orders' tends to have the force of holy writ, it is now accepted as potentially criminal if people follow orders they know should never have been given. That is what the Nuremberg trials of the 1940s were about. Knowledgeable whistle-blowers have a duty to blow their whistles when the circumstances are appropriate.

On that score, the confirmation by the Paris courts last week of last year's convictions of Dr Michel Garretta and Professor Jean-Paul Allain for their part in the scandal of blood contaminated by HIV was a foregone conclusion. Both knew that the blood-clotting factor (factor VIII) they were supplying to haemophiliacs in France between 1984 and October 1985 was contaminated by HIV, but they did not blow their whistles.

So far, so clear and simple. But it does not follow that justice has been done. To be sure, Garretta and Allain could each, separately, have blown a whistle at some stage between the beginning of 1984 and October 1985, perhaps leaking the truth about the contamination of French transfusion blood to the press or even telling the well-organized association of haemophiliacs of what they knew. As they cool their heels in jail, each of them probably regrets that he did not do just that. But the responsibility (as distinct from the right) to break ranks with their organizations laid on whistle-blowers cannot be absolute. Justice also requires that some regard should be paid to extenuating circumstances. Who else knew, and might more properly have blown a whistle? What were the pressures to conform with the mistaken policy to which potential whistle-blowers were exposed? What, if any, was the protection available to those who offended their organization?

Although the French courts have now held two full-scale trials of Garretta, Allain and their two co-defendants, these questions remain unanswered. For one thing, the peculiar circumstances of the National Centre for Blood Transfusion (CNTS) in 1984 and 1985 seem not fully to have been taken into account. The organization, previously an arm of the health ministry, had just been turned into a kind of public

foundation; it was supposedly autonomous, and expected to cover its costs by the sale of transfusion blood and other blood product. To help it on its way, it had been given a monopoly of imports of blood and blood products into France. That helps to explain the recurrence of the chilling phrase "while stocks last" in the evidence that Garretta approved of the continued supply of contaminated products. By the evidence of the trial, Garretta, a long-standing employee of CNTS, was a better administrator than scientist. It is a lot to ask that a person newly put in charge of a new organization should rock the boat while the ink of his letter of appointment is hardly dry.

Allain is a different case. He was better placed to judge the risks of distributing contaminated blood to haemophiliacs; his defence is that he did protest, but internally, to Garretta and to others among his colleagues. It is also a matter of record, and the chief count against him at the second trial, that he failed to tell the haemophilia association in plain language what risks its members were running. To have done so, of course, would have been to flout the decision of CNTS that contaminated blood should continue to be supplied, even if only to those haemophilia patients already contaminated with HIV. There would have been a great row. Whether the protection the haemophilia association could have summoned up would have enabled him to keep his job, or any job, must have been an open question.

For the real difficulty was the climate in which both men found themselves. Autonomous or not, CNTS was still dealt with by the health ministry as if it were still a part of the public service. Strategy meetings were still convened by the ministry. Naturally enough at the time (but not with hindsight), the government's influence was in favour of postponing the heat-treatment of contaminated blood and even the screening of blood donors until French technology became available. It now emerges that that was a shameful influence; but can it be fair that those who exerted it have not been arraigned (if only for the sake of completeness) alongside those now sent to jail?

The lessons of this trial for the research community are therefore profound. What will happen to researchers working for, say, a pharmaceutical company if they know of data that contradict a decision to put a novel medicine on the market? By the Garretta-Allain test, they must blow the whistle, whatever the consequences. What of an employee of a defence research establishment who knows that some new weapon is unsafe, or offends against one of the interna-



tional conventions limiting the kinds of horrors that may be perpetrated even in warfare? He, too, has a duty to say so aloud, whatever the consequences. The obvious difficulty is that there is no general protection (although, in the United States, there is an arrangement that those who spot financial irregularities in the doings of defence contractors may be compensated with money). But such conflicts of interest between an employee and his organization are, in all probability, common. Who, but the research community, can demand, perhaps even offer, the protection that potential whistle-blowers need? □

Europe's water-wars

The European Communities need more rational ways of setting environmental standards.

THE European Communities (EC), which may yet become in the coming months the European Union that Maastricht would make them, are plainly heading for a corrosive internal squabble about the cost of pure water (see page 270). The issue is simple enough. The member states have accepted proposals by the European Commission for the purity of drinking water, and that off beaches, but some of them are now sucking their teeth at the extra costs. This will not be the first occasion when national governments' officials, straight off their early morning flights to Brussels, have put their names to documents whose implications they do not understand, and which often have not been calculated. But the impending row about the cost of pure water raises important questions about the way in which Brussels functions.

Formally, the European Commission's right to insist that the EC should have uniform standards of water quality (and of many other environmental variables) stems from the Treaty of Rome, and from the requirement that no member state should give its manufacturers an unfair commercial advantage in the single European market by avoiding environmental costs to which others are subjected. On the face of things, that seems only fair. But the doctrine of the level playing field does not help decide what particular standards should apply to drinking water, or air quality or the contamination of foodstuffs by, say, pesticides. Often, as with the allowed concentration of nitrate in drinking water, they are more stringent than required to avoid identifiable dangers. The reasons why matters have turned out like that are simply understood: stringency in setting standards always seems more virtuous than laxity.

The error in this way of working is that it can be uneconomic, perhaps ruinously so. Good sense, on the other hand, would argue that commonly enforceable standards should be as lax as is consistent with the avoidance of known risks. Such a policy would not of course prevent EC members (or states in North America such as California) setting higher standards for themselves, but such decisions would be recognizable for what they are: decisions to make public purchases of higher environmental quality on behalf

of a subsection of the union. That is the direction in which Europe should now move. The distinctively European doctrine of 'subsidiarity' (which means delegation) allows for that. More urgently, it will make no sense that the European Union should be hamstrung economically by unduly stringent standards while having to redistribute internal revenues in favour of members that cannot afford them. □

Ol' man river again

The tragic floods in the Middle West are not biblical, but consequences of an immature drainage system.

THE floods in the Middle West of the United States are not simply proof that Mark Twain's awe of the Mississippi was well placed, but a tragedy for the hundreds of thousands of those directly affected by the floods. When typhoons roar up the Bay of Bengal and drive even greater numbers from their more primitive homes, the television cameras are rarely as well placed as they have been in the past two weeks, and the tragedy is more quickly forgotten. But, luckily for North America, even the awesome Mississippi is more tractable than the typhoons that regularly hit Bangladesh.

The place to start is with a recognition that the Middle West is what it is, and that it is able to boast of being the world's granery, because it lies in a region from which the postglacial ice has disappeared only relatively recently, within the past 8,000 years. Despite (or, rather, because of) the scale of the Mississippi drainage system, which carries away the rainfall between the Rockies in the West and the Appalachians in the East, the river system is immature in geomorphological terms. The way in which the Missouri River seemed last week to have broken through to the Mississippi channel some 20 miles north of its normal confluence is proof of that. But the thick layers of black soil that have made a bread-basket of the Middle West is also a proof of the frequency and the scale on which the river system floods.

In all the circumstances, the much-derided US Army Corps of Engineers has done well, over several decades, to contain the damage done by all but exceptional floods — this year's, for example — even if its levees should help to keep floodwater in place for some weeks to come. What role it or some other agency should have in the months ahead will nevertheless depend on what kind of river is left when the floods go down. Many of the levees, tall though some are, will have disappeared. Should they be rebuilt, and at whose expense? It may prove to be a better strategy to resite damaged dwellings and farms in such a way that they may escape future floods. Heavy engineering, if it has a place of any kind, would be better used in speeding the discharge of water along the stretches of river not in flood. Even the intelligence and resources that North America can muster will not contain all future floods, but they should go a long way to minimize the disruption caused in the past few weeks. □

French appeal court sends Allain to jail

Paris. Two former officials of the French National Centre for Blood Transfusion (CNTS), Michel Garretta and Jean-Pierre Allain, last week lost their appeals against four-year prison sentences. They were convicted last year of consciously allowing haemophiliacs to be treated in 1985 with clotting factors contaminated with HIV when a heat-inactivated alternative was available.

The appeals court upheld the four-year sentence for Garretta, former director-general of CNTS. It reaffirmed that he knew CNTS clotting factors were contaminated and that one-tenth of haemophiliacs treated would die of AIDS within five years and that he continued to supply contaminated clotting-factors "while stocks last" for financial reasons, instead of importing heat-inactivated preparations. (CNTS had a monopoly for the import of blood products into France.)

The court also upheld the four-year sentence (with two years suspended) for Allain, former head of research and development at CNTS. It convicted him, as a "specialist of haemophiliacs", for not telling haemophiliacs that all stocks were probably contaminated, for being at the meetings at which CNTS chose to supply unheated clotting factors "while stocks last" and for continuing clinical trials of untreated products after he knew of the danger.

Robert Netter, former head of the National Health Laboratory, who was previously acquitted, was given a one-year suspended sentence. Jacques Roux, former director-general of health at the National Ministry of Health, had his four-year suspended sentence reduced to three.

Olivier Schnerb, the lawyer representing Allain, says it is "abnormal" that the appeals court should have convicted Allain on "new grounds" after having rejected those on which he was first convicted: that he was present at the meeting in May 1985 which decided to delay introduction of heat-treated products for five months. Schnerb will fight the verdict in the Supreme Court of Appeals.

Schnerb now alleges that the trial was a "decoy" to "appease public opinion" and to detract attention from another "bigger scandal": that a deliberate delay — this time in screening blood donors for HIV — exposed the wider public to risk of HIV infections from transfusions with contaminated whole blood. This affair has been hushed up, he claims, because it could "implicate former ministers, and their chief scientific advisers". Government officials are alleged to have deliberately delayed approval of a US AIDS test for five months in 1985 so that a test developed by Diagnostic Pasteur could be released first (see *Nature* 353, 197; 1991).

"It's so damn obvious that this question

comes next," says Jacques Liebowitch, of the Raymond Poincaré Hospital near Paris, "it's amazing it didn't come up before." More than 9,000 people were infected with HIV in France following transfusion with contaminated whole blood, he says, many during the five-month delay before France began systematically screening donors.

Using a prototype indirect immunofluorescence AIDS test, Liebowitch and François Pinon of Cochin Hospital in Paris discovered in December 1984 that one in every 200 blood donors in Paris was seropositive. "We said that donors needed to be screened immediately," he claims, "but there was a plot to play down the risk of blood contamination and the pathogenicity of the virus, because of the alternative Abbott (US) test . . . this propaganda was spread right up to the level of the prime minister . . . it was passive mass murder."

One reason why infection by whole blood transfusions has attracted little attention is that individuals have not called for criminal proceedings: most have settled for compensation of several million francs. Judicial proceedings were begun in the clotting factors case only after persistent efforts by the well organized haemophilic groups.

"Protected" individuals have escaped justice, Schnerb says. He claims that this also explains why Allain was originally tried in a magistrate's court on the trivial charge of deception over product quality: "They wanted a scapegoat, but keep clear consciences by giving him a short sentence."

Fight to relax German research curbs

Münster. The Deutsche Forschungsgemeinschaft (DFG), Germany's major sponsor of basic research, plans openly to fight back against the public hostility directed against German researchers, which it believes is not found elsewhere in Europe.

At DFG's annual meeting earlier this month, it was decided to establish a working group specifically aimed at countering "obstructions to research". The initiative is the first of its kind in Europe.

President Wolfgang Frühwald told the gathering that it is "high time to challenge the infectious spread of anti-scientific sentiment in Germany", to which he partly attributes Germany's falling international competitiveness in some fields, notably in information technology and biotechnology.

The working group will take on all issues that impede research, particularly in the areas of genetic engineering, data exchange, embryo research and animal experimentation, where legislative "safeguards" consid-

Judicial reform

Meeting last Monday (19 July) at the Palace of Versailles outside Paris, for only the fifth time since the birth of the Fifth Republic in 1958, the French Parliament voted for changes to the constitution — a reform of the High Court of Justice — that could speed up the prosecution of former ministers allegedly involved in the blood contamination scandal.

Previously, current and former cabinet officials could be judged only by the High Court of Justice, and only if the charges were brought by a member of parliament: the court was never convened during the Fifth Republic. Clamour for change began last year, following the failure of repeated attempts to try three Socialists in power at the time of the blood scandal — former prime minister Laurent Fabius, the former deputy health minister Edmond Hervé and the former social affairs minister Georgina Dufoix.

The key change is that anyone can now bring charges against current or former ministers. These would be judged not by the High Court but by a newly created lower court, the 'Court of Justice of the Republic'. The High Court will now exist only to judge the president for high treason. **D.B.**

He also argues that Allain should have been tried in a criminal court, where it would have been necessary to decide whether he was "innocent or guilty". "Only four years for a death! That's stupid. It should be either a life sentence or acquittal."

Declan Butler

ered over-stringent cause researchers to abandon the bureaucratic battle - and their experiments. For example, Germany's gene law (which may be somewhat relaxed before the end of the year) has made it almost impossible to do any experiment at all that involves gene manipulation (see *Nature* 363, 481; 1993). DFG fears that these frustrations could drive promising young researchers abroad.

The new working group probably will be headed by Rüdiger Wolfrum, DFG board member and director of the Max Planck Institute for Comparative Public Law at Heidelberg. The composition and work-plan of the group has not yet been defined, but DFG hopes that other research organizations in Germany, such as the Max Planck Society and the organization for the national research centres, will eventually join in.

Robert Unterhuber

■ "Germany's tight budget", page 273

Clash over too demanding Euro-water standards

London. Complaints that European standards for drinking water are uneconomically over-stringent are mounting. In Britain, where water utilities in England and Wales have been privatized in the past two years, the head of the regulatory body that oversees the prices they now charge has warned that customers may not be willing to meet the costs resulting from legislation aimed at improving the quality of drinking water.

The warning comes from Ian Byatt, director-general of the Office of Water Services (OFWAT), just as the European Commission in Brussels is preparing to negotiate

cide rules based on guidelines prepared by the World Health Organization. They specify limits based on observed toxicity of individual chemicals rather than sensitivity of detection techniques. In most cases, upper limits are 0.2 micrograms per litre.

But any move to relax water quality regulations will be fiercely resisted by environmental groups. Last week, Friends of the Earth (FoE) in London claimed that delays in the introduction of the 1975 regulations in Britain meant that "at least 14.5 million" people are still drinking "sub-standard" water. At the prompting of the FoE, the EC environment commissioner has told Britain that it is not satisfied with its explanation for delay, and is considering referring the matter to the European Court of Justice.

The pressure groups also have support in Brussels for their claims that complaints of excessive regulation are motivated as much by concerns about the profitability of the water companies as about the effectiveness of the services provided to customers. Ioannis Paleokrassas, the EC environment commissioner, has already dismissed OFWAT's criticism of EC directives, arguing that the real failure has been Britain's reluctance to enforce the "polluter pays" principle on its farmers and waste-producing industries.

Even if the water industry manages, as seems likely, to persuade the European Commission to relax the rules on water quality, a political problem remains. Any proposed changes would need to be unanimously approved by all member states.

Thus the main thrust of OFWAT's advice to the British government is that it should seek to mitigate the impact of future regulations in the process of being drawn up, rather than to cut back on those already in force.

OFWAT warns that water bills are likely to increase five per cent faster than the expected rate of inflation between 1995 and 2000, with some customers facing an increase of up to £100 on bills that which now average £100 a year.

Even if the EC does agree to change the most heavily criticized standards, such as those for nitrates and pesticide residues, new regulations are unlikely to be in force quickly enough to affect the short-term investment plans of the water companies.

Thames Water Utilities Ltd, for example, suppliers of most of London's water, is already committed to spending £380 million over the next three years to reduce pesticide residues to the existing EC limits. "We are all lined up to meet the standards being required of us; it's just that we don't want any more like that", says a company spokesman.

David Dickson

SSC supporters hope for a last-ditch reprieve

Washington. Supporters of the Superconducting Super Collider (SSC) are asking the US administration to pledge support for a future particle accelerator in East Asia in exchange for support now to save the troubled Texan project.

The House of Representatives last month voted down funding for the project (see *Nature* 364, 6; 1993), but its supporters think it could yet be saved by agreement between the House and the Senate. With this in mind, George Brown (Democrat, California), chairman of the House Science, Space and Technology Committee, wants the administration to promise Japan and other Asian nations three things in return for some financial support — a "full voice" in project management, an allotted share of science time and a pledge to help build the next-generation particle collider in Asia.

"Now is the time to establish a fully international framework for high-energy physics", says Brown, who wants to see Europe, the United States and Asian countries join in such a framework in time to save the SSC. "Obviously this will look like a desperate plan to keep the Super Collider alive...but in my own defence ... I've always said the SSC should operate this way."

Brown says such a framework would involve no exchange of money between Europe and the United States, but a commitment from both groups that the world's next great accelerator would be built in East Asia, in exchange for financial support now. The only possible source of funds sufficient to save the SSC is Japan, but the new framework might also interest Hong Kong, South Korea, Taiwan, Australia and some smaller countries.

"A commitment from them, backed up with a small amount of money this year" would suffice to get the new arrangement off the ground, Brown says. He has discussed his plan with John Gibbons, President Clinton's science adviser, but received only a vague assurance that it was worthy of further exploration.

But Japanese officials are sceptical, and question the ability of the US administration to deliver a long-term promise, given congressional constraints. "Certainly if other countries promise to build the next collider in Japan, it helps us to contribute to the SSC", says Akihiro Maki of the Japanese Society for the Promotion of Science Washington office, but he doubted whether "any US administration can make such a promise".

The Japanese say that if Clinton is serious about saving the SSC, he should make his own commitment to it clear, and make a formal request for support to their government.

Colin Macilwain

IMAGE
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Thames Water's Tony Rachwal with the fine carbon for the Thames Superfilter, promising "crystal clear drinking water."

a possible revision of the water quality standards set in 1975, which are now mandatory in all member states of the European Communities (EC).

Changes in current standards, particularly those specifying maximum levels of nitrates and pesticides, are being demanded by Europe's water industry on the grounds that they are too demanding and also "unscientific". The industry argues that the current nitrate limit of 50 micrograms per litre, for example, based almost entirely on the avoidance of infantile methaemoglobinemia ("blue baby" disease), takes no account of its virtual disappearance where public supplies of water are treated.

Europe's water companies, organized through a trade association known as EUREAU, also want the limits on pesticide residues rewritten. These fix an upper limit of 0.1 micrograms per litre for individual pesticides, or 0.5 micrograms for all pesticides. Given the detection techniques of 1975, these were then regarded as a "surrogate zero".

Backed by several EC members including Britain, the companies want new pesti-

Earthquake exposes gaps in seismic network

Tokyo. The devastating earthquake and tsunami that struck the coast of northern Japan last week, killing more than 200 people, has drawn attention to gaps and duplication in Japan's seismic networks used for earthquake prediction and disaster prevention.

Last week's was the second major earthquake in the Japan Sea in recent years; on 26 May 1983, another struck a few hundred kilometres further south and killed 104 people, again largely because of the accompanying tsunami. Both earthquakes occurred in a major fault zone which has nevertheless not been designated as one for special monitoring and protection under Japan's earthquake countermeasures law.

The earthquake, measuring 7.8 on the Richter scale, struck in the middle of the night just north of the little island of Okushiri, off the northern island of Hokkaido. Most of the 4,700 inhabitants quickly realized the threat from tsunami — several of the island's houses were washed away by tsunami from the more distant 1983 earthquake — and so fled to higher ground.

Others were not so lucky. The shorefront Yoyoso Hotel on Okushiri was struck from behind by a landslide and then from the front by a series of tsunami, the first about five minutes after the earthquake. The tsunami reached a maximum of 30 metres above sea level in the southwest part of the island, according to preliminary measurements by scientists from Tokyo and Tohoku Universities. That would make the tsunami the highest to have hit Japan since a 38-metre tsunami killed 22,000 in the Sanriku region in 1896.

The town of Aonae on the island's southern tip was devastated last week. Half of its 700 houses were destroyed by an uncontrollable fire caused by a ruptured propane tank.

Tsunami also caused extensive damage on the east coast of Russia and Korea, but there seem to have been few casualties.

There have been six major earthquakes since 1940 in the seismically active fault zone, which runs parallel to the west coast of Japan. The poor understanding of the region is attributable to a lack of seismograph stations, according to Hideki Shimamura, of Hokkaido University's laboratory for ocean bottom seismology.

Along the Pacific coast and within about 200 km of Tokyo, there are 133 observation instruments, including 54 land-based seismographs and 8 ocean bottom seismographs run by various government organizations, but that there are only 9 seismograph stations along the 400-km Japan Sea coast of Hokkaido.

This week, Shimamura hopes to deploy 10–15 ocean bottom seismographs in the region around the earthquake's epicentre, using a Japan Meteorological Agency ship for the purpose. Temporary seismographs

have also been set up on Okushiri. But more permanent stations are clearly needed in the region.

Under Japan's Large-scale Earthquake Countermeasures Act, ten regions have been designated for intense observation, but the list has not been revised since 1978 and the



active fault-zone in the Japan Sea is not among them.

Kiyoo Mogi, chairman of the coordinat-

ing committee for earthquake prediction, announced last week that the committee would consider designating the region as one for more intense observation in its next five-year plan, due to be published later this month. But designation is highly political because it can depress land prices and drive up the cost of insurance. Equally, it is difficult to remove regions already designated because that would mean loss of government subsidies for protection against tsunami and earthquakes.

In any case, designation does not necessarily result in an efficient network. In the Kanto and Tokai regions around Tokyo, six different government ministries and agencies conduct seismological, geodetic and geological observations, sometimes from separate stations barely 50 metres apart. Government bureaucracy limits the exchange of data between different government agencies and data are not made quickly and freely available to researchers outside the prediction programme. Despite complaints by Earth scientists outside the prediction programme, the now-elderly scientists who set it up seem determined to keep it on its 30-year-old course.

David Swinbanks

No major changes in earthquake prediction

Tokyo. Despite severe criticism, Japan's earthquake prediction programme seems unlikely to be shaken up by last week's earthquake in the Japan Sea.

The programme was formally reviewed last year, at the end of its sixth five-year phase, when outside opinions were sought for the first time. Several researchers criticized the programmes' objectives and organization; it has consumed about a billion dollars and engaged about 500 researchers over the past 20 of its 30 years (see *Nature* 356, 464; 1992).

The external review was never made public, but some who attended the hearings

in May last year say that some of the reviewers, who included Saburo Nagakura, president of the Graduate University of Advanced Studies, were very critical and even questioned the premise that earthquake prediction is possible.

But those who have seen the plan for the next five years say that "only cosmetic changes in wording" have been made, placing more emphasis on "basic research". They also say there is no fundamental change in the direction of the programme, which concentrates on collecting empirical data in the belief that one day a reliable precursor of earthquakes will be found.

The committee has some things to say in its own defence. Kiyoo Mogi, chairman of the earthquake prediction committee, suggested last year that a new type of "grey" prediction should be introduced, allowing the committee to say that an earthquake "might" occur. But that idea has been rejected by government bureaucrats, who have written laws specifying that the committee must give black or white decisions.

David Swinbanks

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Aonae engulfed in flames — with ships washed up by tsunami.

British research councils win and lose

London. Britain's Science and Engineering Council (SERC) seems to have been the chief loser in a redistribution exercise ahead of the shift from five to six research councils due next year.

Notably, SERC has lost its bid to keep bioengineering under the same roof as other branches of engineering. Proposals put to the government last week on implementing June's white paper (policy document) on science would transfer most research into the engineering dimensions of biotechnology to the new Biotechnology and Biological Sciences Research Council (BBSRC).

That is a victory for Tom Blundell, the chairman of the Agriculture and Food Research Council, who has argued strongly for an expanded role for his council. Under its new name, the council will be responsible for research ranging from basic biology to its industrial applications, and will as a result see its budget rise by 40 per cent, from £117 million (\$175 million) to £167 million.

That will disappoint the Science and Engineering Council, due to split into two organizations: the Engineering and Physical Sciences Research Council (EPSRC) and the Particle Physics and Astronomy Research Council (PPARC). While accepting the idea of a research council spanning basic and applied biology, SERC officials had urged that bioengineering should keep close links with other engineering disciplines.

"The government has been keen to set up a credible biotechnology council, but its efforts have perhaps been at the expense of engineering, since it does not seem to be concerned about doing something equally credible for engineering research", Sir Mark Richmond, the chairman of the SERC, said this week.

The so-called "boundary study" is by Sir David Phillips, who remains chairman of the Advisory Board for the Research Councils until the end of the year, but was in this case acting as Director General (DG) for the Research Councils, a new position whose creation was one of the key recommendations of the white paper.

The main thrust of the study has been to identify specific areas of responsibility for the six new research councils (see figure),

each of which will be expected to fund both basic and applied research considered germane to a clearly defined mission. Biotechnology, occupying a grey area between the life and the physical sciences, has proved one of the most troublesome issues.

One of the few compromises is on biomolecular science, which chemists had feared might be absorbed into the new research council. According to a draft of

ity" for supporting research unable to find a natural home — a task previously taken on, not always willingly, by SERC.

Concern over whether the new director general will have sufficient authority and influence to prevent important research from falling through the cracks was expressed last week by several members of the House of Lords Select Committee on Science and Technology. "Put bluntly, the DG must

have clout", said Lord Dainton, who chaired the committee's recent inquiry on the neglect of taxonomy.

William Waldegrave, the minister for science, reassured the committee that the new post was a "key role", since its occupant would be required "to shape the direction and strategy of the research base". Waldegrave said that the research council heads will continue to report directly to the

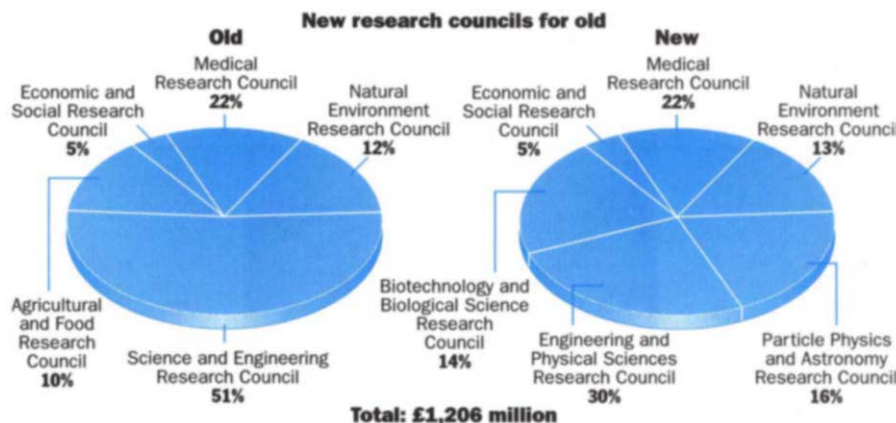
minister (and not through the DG). But he defended this decision on the grounds that the minister would rely on the DG to check that the research councils were fulfilling their responsibilities, and "are not just paying lip service to their mission statements".

David Dickson

■ The Office of Science and Technology is to organize seven regional seminars in different parts of Britain over the next few months to generate support for its "technology foresight" programme. That initiative, launched in the white paper, aims to build consensus on the key generic technology of the future, and on the orientation of the research community's efforts to those ends.

Waldegrave this week told a meeting of the Confederation of British Industry, which enthusiastically supports the foresight initiative (and will help to organize at least two of the regional meetings), that the approach is similar to that successfully applied by the Japanese government to identify areas requiring greater research effort.

Waldegrave said he wanted to use the technology foresight initiative to build "more intimate, regular and structured contact between the scientist and engineer at the bench and the industrialist". He hopes the seminars will "develop a commitment to the programme" across all sectors of the community; industrialists, academics, consumer bodies and representatives from the public sector will be invited to them. □



Phillips's conclusions, there will instead be a joint committee between the BBSRC and the EPSRC, with funds placed in a common pot by the two bodies — an arrangement meant to protect resources at present allocated to chemistry.

SERC had been hoping for a similar compromise for biochemical engineering, but Phillips came down clearly in favour of shifting that to the new biotechnology council, on the grounds that research in that field will contribute to BBSRC's role in supporting Britain's biology-based industry.

An early draft of the boundary study also suggested that funding for biosensors research be allocated to the engineering council, on the grounds that it would "sit more comfortably" next to molecular electronics and other sensor technologies. But Phillips is reported to have changed his mind, and to be suggesting in his final report that this too should go to the biotechnology council.

A less controversial Phillips proposal is that responsibility for Earth observation, atmospheric chemistry and science-based archaeology, previously funded by SERC, should be moved to the Natural Environment Research Council (NERC). He points out that the move puts responsibility on the NERC for developing the technological support required by each of these areas.

Providing clear mission statements to each of the research councils will, as Phillips acknowledges, mean that none will have the role of "residual responsibility"

Germany's tight budget

Munich. Germany's belt-tightening 1994 budget, announced last week, precludes overall growth in science. But the prospects could have been a lot worse.

The research ministry's share of the federal budget for 1994 will be restricted to around DM9.5 billion (US\$5.9 billion), the same as in the current year. But research organizations are relieved that the 'five-times-five' agreements, which promised five per cent annual growth from 1991 to 1995, will not be broken. And there will be no back-peddalling on promises to maintain the high level of support to the new *Länder*.

Research minister Paul Krüger, an east German in office for only three months, has had the task of sharing out a budget with zero increase while inflation is running at 4.5 per cent. The ministry has already committed itself in principle to bolstering research in new technology and building up the research infrastructure in eastern Germany; Krüger says this can now be achieved only by making research 'more efficient'. Although 'efficiency' tends to be a euphemism for 'cuts', in this case the distribution of the reduced budget will probably be welcomed by all except those whose work is directly affected by shifts in priority.

The areas hit include conventional energy research and flight and marine technology. But the budget also raises the prospect of reduced future contributions to international enterprises such as the European Space Agency (ESA), which receives 23.6 per cent of its budget (this year DM1.2 billion) from Germany. The federal government says it

plans to open negotiations with its partners, acknowledging that such steps will require the cooperation of all ESA member states.

But not all is gloom. Krüger says that biotechnology and transport technology, for example, will receive more support (2.3 and 4.0 per cent respectively), as will health research (3.3 per cent), ecology (2.2 per cent) and climate research (1.8 per cent). All figures are, however, below inflation. Information technology remains a high priority, but Krüger is letting the European Communities shoulder that burden, and hopes to reduce the ministry's contribution in that area. Similarly, there are no extra funds for environmental research, where Krüger wants research institutes to take responsibility.

The major pure and applied research organizations outside the universities, the Max Planck Society and the Fraunhofer Society, will be given their promised five per cent increases, but — as also promised — the national research centres will have no increase over 1993. These large-scale centres, widely regarded as particularly inefficient and inflexible, have been under pressure for the past few years.

But eastern Germany is the clearest winner, if such a term can be applied to the underprivileged new *Länder*. True to his word, Krüger is allocating an extra DM100 million (US\$62.5 million), an increase of 13.7 per cent, to support research and its infrastructure in the region. Part of this money will be given to "promote cooperation between university research and industry". Although generally fashionable in Europe,

this concept may not be easily implemented in an environment where research is still not properly reintegrated into universities.

The Deutsche Forschungsgemeinschaft (DFG), which sponsors research in universities, also benefits from the five-times-five agreement, to which its sponsor, the education ministry, has adhered. But education, with a four per cent reduction from this year's budget, has fared even worse than research. The sharpest reduction will be in the budget of the body responsible for student grants, which will be reduced from DM2.55 billion for undergraduate support this year to DM2.3 billion next year; parents will be expected to bridge the gap. And despite chronic overcrowding, there is no increase in the university building fund.

Alison Abbott

New NSF director nominated

Washington. President Bill Clinton has nominated Neal Lane, an atomic physicist and provost of Rice University, Texas, as director of the National Science Foundation (NSF). If his nomination is confirmed, Lane,

who is 54, says his main challenge at the \$3 billion agency will be "to set priorities that will promote progress in science and engineering".

A member of the Democratic Party whose wife, Joni Sue, is active in party politics, Lane



Neal Lane, facing the "squeeze"

says: "I know the administration is strongly committed to research and education, so I had no reservations about accepting".

A native of Oklahoma and a long-time physics lecturer at Rice, Lane briefly worked for the NSF as director of the physics division in 1980. He has also spent some time at Queen's University, Belfast, and at the Joint Institute for Laboratory Astrophysics in Boulder, Colorado, and was chancellor of the University of Colorado during two financially difficult years from 1984.

Lane says he acknowledges that many researchers "do fear being squeezed" as research becomes more expensive. President Clinton has requested a 16 per cent increase in NSF for the coming financial year, although Congress is unlikely to appropriate all of it.

If confirmed, one of Lane's first tasks will be to help to decide the outcome of a review of NSF's large supercomputer centres, which were set up in 1985 on the recommendation of a panel that he chaired.

Colin Macilwain

Hasty exit from APS

Washington. Mystery this week surrounds the departure of Richard Werthamer, erstwhile executive secretary of the American Physical Society (APS), who was suddenly



Richard Werthamer (left) and Harry Lustig

and unexpectedly ousted last week from the main administrative job at the 43,000-strong professional grouping.

Several of APS's élite band of general councillors said last week that they were unaware of Werthamer's departure, as indeed was Werthamer himself. It was "a rumour I'm not aware of", he said on

Wednesday last week. But by Friday he had left the Manhattan headquarters of APS, which issued a bland statement citing "differences concerning APS management policy and practices" as an explanation for his "resignation".

Members of the APS council which removed Werthamer, including president-elect Burton Richter of the Stanford Linear Accelerator Center in California, referred enquiries to the president, Don Langenberg of the University of Maryland. But Langenberg was not returning calls.

Werthamer, who joined APS in 1991 from a medical instrument company, was engaged in the delicate process of moving APS's large publishing staff from New York state to new premises on Langenberg's Maryland campus, a move still due to take place in October. He will be replaced temporarily by long-time treasurer Harry Lustig, while a panel under past-president Ernest Henley searches for a new executive secretary.

Colin Macilwain

US shows good faith on help for developing countries

Washington. One of the most vivid signs so far of the altered US stance on global environmental problems came last month, during the first session of the United Nations Commission on Sustainable Development.

The US delegation announced a partnership with Colombia on one of the most tangible goals outlined at the 1992 Rio conference: the exchange of environmental technology from developed to developing nations. In the coming year, officials from both countries will attempt to organize the transfer of technology for eco-efficient manufacturing, energy and transportation systems and sustainable agriculture and land use to developing countries.

At one level, the joint effort is symbolic, marking an about-face from former US President George Bush's obstructionist image at the Earth Summit. Colombia is the current head of the Group of 77, the coalition of 129 developing countries which has criticized the United States for environmental hypocrisy and for ignoring their right to develop as they see fit.

The partnership with Colombia is the latest in a series of moves by the Clinton administration to put its stamp on environmental politics worldwide. On 4 June, the United States signed the biodiversity treaty after having been alone among large coun-

tries in refusing to do so. A month earlier, the US delegation pledged \$100 million to fund international population planning, reversing the 12-year policy of the Reagan and Bush administrations.

In April, President Clinton announced that the United States would go beyond the Bush policy on global warming by reducing emissions of greenhouse gases such as carbon dioxide to 1990 levels by 2000.

Perhaps the most significant change in White House policy came with the acknowledgement that the US contribution to the degradation of the global environment has been disproportionately great. "Sometimes the developing countries are right...The affluent of the world have a responsibility to deal with their disproportionate impact," said US Vice-President Al Gore in his opening speech to the UN Commission.

Although some delegates from developing nations feel the United States is doing "too little, too late," many seemed encouraged by the new administration's change in policy. Tony Lambe, who coordinates the efforts of nongovernmental organizations' (NGOs) towards sustainable development for the body called Earth Council, says that while "it's still uncertain what Clinton will ultimately accomplish", the United States is "certainly making the right noises".

Process patent bill on track

Washington. A new law that would afford greater patent protection for US inventions in biotechnology and place the United States on a more equal footing with Europe and Japan inched one step closer to enactment last week, after receiving unanimous support in the US Senate.

As in past years, when the same bill was introduced but not enacted, Congress has to decide whether to tinker with US patent law and overturn a 1985 Federal Circuit Court ruling, *In re Durden*, which suggests that the use of a novel starting material in combination with a known chemical process is not eligible for process patent protection.

Supporters of the bill claim that over the past seven years, the US Patent and Trademark Office has erroneously and inconsistently used *Durden* either to delay or deny biotechnology process patents. This, they say, puts the United States at a considerable disadvantage in the face of competition from countries such as Europe and Japan, which routinely grant process patents.

Co-sponsored by Senator Dennis DeConcini (Democrat, Arizona) and Representative Rick Boucher (Democrat, Virginia), the Biotechnology Patent Protection

Act would not only amend existing US patent laws to include patent protection for genetically engineered processes when the combination of a novel starting material and a known process produces a useful product but would also close a legal loophole that allows foreign companies to export to the United States biotechnology products produced using components (such as host cells) patented in the United States.

Taken together, Boucher says that the bill would form "a comprehensive solution to the problem". But the passage of either of the parts independently would still be an effective solution, he says.

Although approved by the Senate, the bill's supporters are not yet in the clear as the proposal has yet to be voted on in the House of Representatives.

Past attempts to overrule *Durden* have been endorsed by the patent office but opposition remains among certain intellectual property groups on the grounds that the bill smacks of special interest legislation and amid concerns that it will lead to the automatic issuance of process patents which would increase uncertainty in the law.

Diane Gershon

The main accomplishments of the first meeting of the commission are thus intangible: US enthusiasm and reduced tensions between rich and poor countries, which exceeded expectations. Although no government formally committed funds to projects, developed countries agreed to encourage debt-relief for heavily indebted developing countries implementing environmental programmes. The commission also agreed that participants will submit annual reports on their progress towards Agenda 21, the action plan outlined at Rio. Environmentalists hope the reports will have the effect of encouraging compliance with Agenda 21, for which there are no formal enforcement procedures. **Susan Greene**

European alarm on intellectual property

Strasbourg. The European Parliament gave qualified assent last month to ratification by the European Communities (EC) of the UNCED (United Nations Conference on Environment and Development) convention on biodiversity, but expressed alarm over the EC's additional clause defending intellectual property rights. The accord, signed last year in Rio, is to be ratified by the 12 EC member states and by the EC, in its own right, by the end of the year.

Some member states argue that the Treaty of Rome grants the EC no powers in the area of intellectual property rights. Parliament also deplored the move by the EC to follow the United States in drafting a unilateral 'interpretive declaration' which adds conditions to the agreement in Rio on sharing research benefits with developing countries.

But the commission insists that the biodiversity convention is partly concerned with trade in intellectual property — and it does have appropriate powers in the field of trade. While reaffirming EC support for the idea of technology and biotechnology transfer, the EC declaration insists that respect for intellectual property rights must be an "essential precondition". Transfers would entail "compliance with the principles and the rules of protection of intellectual property, and in particular multilateral and bilateral agreements signed or negotiated by the contracting parties to this convention".

This implies, complain parliamentarians, that patent rights are more important than the countries' sovereign rights over natural resources. Strasbourg urged that the declaration should be dropped, leaving North-South negotiations on an equal footing. It can, however, do no more than urge. Pre-Maastricht, parliament has in fact little power to counter the combined weight of a common council and commission position. And EC commissioner Manuel Marin made it clear that he would not be shifted from his pro-council position, now likely to be ratified quite soon. **Arthur Rogers**

World Bank espouses public health for those in poverty

Washington. The World Bank intends to redirect much of the \$350 million it spends each year towards preventive medicine for the nearly one billion people living in poverty worldwide.

This decision is embodied in a report, *Investing in Health*, that emphasizes the importance of basic and cheap public health measures not only in helping people live longer happier lives, but also in increasing the productivity of developing countries.

The report argues for doubling or tripling spending on immunization, dietary supplements such as vitamin A and iodine and the prevention and treatment of malnutrition, tuberculosis, maternal deaths, AIDS and other sexually transmitted diseases. The study shows that, since 1950, such efforts in poor nations have decreased infant mortality by two-thirds and raised the average life

expectancy from 40 to 63 years, lifting the economic burden of unhealthy workers and sick or absent schoolchildren in the process.

The benefits of generic rather than brand-name drugs, and of lower-level clinics rather than hospitals, are also urged. In some countries, a single teaching hospital can absorb 20 per cent or more of the government's health budget, even though nearly all cost-effective interventions are best delivered at lower-level facilities.

But the World Bank warns that its recommendations will probably be opposed by "health workers...drug companies, medical equipment manufacturers and other suppliers" on financial grounds. However difficult to implement, the report marks a reaffirmation by the bank of the importance of health care in economic development.

Susan Greene

EC seeks ban on milk yield hormone

London. Despite being given the green light by both health and bioethics experts, bovine somatotropin (BST) — the genetically engineered growth hormone that can increase milk production in cattle by up to 20 per cent — is to likely to be banned in Europe for a further seven years.

The European Commission has recommended to the member states of the European Communities that they extend the current moratorium on BST when it expires at the end of the year, arguing that it would be impractical to impose the safeguards that its advisory committees had recommended. This follows a decision three weeks ago by

the US Senate to impose a 15-month moratorium on a product that has been fiercely attacked by environmentalist groups.

Europe's animal health industry has been pushing for the moratorium to be lifted, concerned that it may become a precedent for other decisions restricting the applications of genetic engineering to animal-based products. But various farmers' groups — including Britain's National Farmers' Union — had opposed such a move, arguing that the introduction of BST could lead to a general decline in public confidence in dairy products.

David Dickson

NIH, FDA seek lessons from hepatitis B drug trial deaths

Washington. Investigators at the US National Institutes of Health (NIH) will conduct a thorough scientific review of all patients who have been exposed to the experimental drug Fialuridine before deciding what lessons can be learnt from a small trial of the drug which left two patients dead and four more waiting for liver transplants.

The NIH trial managers were this week meeting representatives of the Indianapolis-based manufacturer Eli Lilly and officials of the Food and Drug Administration (FDA) to discuss details of the Fialuridine trial itself, and the talks were likely to broaden out to deal with the wider implications for trial design for antiviral drugs.

The potential of Fialuridine as a treatment for hepatitis B was first observed when the drug was being tested on 40 HIV patients. It appeared to have a good effect on patients who also had hepatitis B, leading NIH to proceed with a trial last summer in which 24 hepatitis-B patients were treated with the drug for four weeks. More than a third of the patients responded well, encouraging Eli Lilly to pay its developer, Oclassen Pharmaceuticals, \$7.5 million for international rights to the drug, while NIH planned a further, six-month trial this spring.

The first ten patients on this trial began taking the drug in early April. The trial was halted on 26 June after two of them became seriously ill with abdominal pain and nausea and then liver failure. The two patients died earlier this month, and four others have now been referred for liver transplants.

Jay Hoofnagle, joint principal investigator in charge of the trial at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), says it is not clear whether anything could have been done differently to prevent the trial going wrong. "It came on so suddenly", he says. "We realized the drug might be toxic, but we thought that by tracking everything carefully we could watch out for it."

James Balow, clinical director of NIDDK, said that one of the main problems was that the metabolism of antiviral drugs of this type in humans was very different from that in animals. "For many of those drugs, the animal tolerance is just striking — the animals seem to tolerate them without blinking an eye." Safety trials on healthy humans, as required by the FDA, do not necessarily show up a syndrome which, in this case, struck patients with liver problems.

Balow says it is too early to draw any general lesson from this case. "Each of these drugs is very distinctive in its toxicity. A moratorium on trials of this class of drug would certainly be premature."

Colin Macilwain

Long journey for large mirror



Munich. The largest mirror in the world prepares itself for its long trip by barge from Mainz (Germany) to Paris along the Rhine, the Channel coast and finally up the River Seine. In Paris the 8.2 m diameter astronomical mirror will be given

a two-year polishing before setting off for Cerro Paranal, Chile. There it will be assembled, along with three similar mirrors, as part of the European Southern Observatory's Very Large Telescope expected to be ready by the end of the century. Despite weighing 22,000 kg, the mirror has a thickness of only 177 mm and is therefore very flexible. This means that its curvature can be computer-controlled.

A.A.

Black paper for science

SIR — The publication of the government's White Paper on science and technology has provided opportunities for serious debate about the status of science in the United Kingdom. Most of the Office of Science and Technology (OST) proposals have been welcomed and an innocuous sense of relief now pervades the scientific community.

For myself, the comfort afforded to the OST paper by the guardians of British science rests like a pea beneath the mattress of our science base. I am a young and naive research scientist fortunate enough to have been 'turned-on' to science. For me science is 'a way of life', and satisfaction comes from the possibility that my research may contribute to man's knowledge. The fact that I feel uneasy and out of date with such an appreciation of science is due to recent unremarkable changes in the philosophy of science, changes that have been given an official credibility in the government's paper.

My interpretation of the White Paper seems to be at odds with most of what has been said or written so far and might be summarized as follows.

Britain is in recession. The government is unhappy. Industry is unhappy. Each would like to blame the other. Each would like the other to help to improve the economic reality. Both have, at last, agreed that a strong industrial/manufacturing base is essential to economic recovery. Manufacturing and industrial competitiveness have been in decline for many decades and the government seems to have pinned the blame for this decline firmly upon academic research. We are told that a lack of direction in academic scientific research, particularly as it applies to industry, is chiefly responsible. The White Paper proposes to remedy this through the implementation of several inherently damaging and prospectively irreparable schemes.

(1) Industry has controlled and continues to control most spending on scientific research and development in the United Kingdom. The present balance of payments speaks volumes on their use of these funds and yet the government believes that industry should now be given the opportunity to influence how academic institutions, through the research councils, spend the remaining part of the research and development budget. (2) Basic science is to be gradually replaced by foresight, a transition that will greatly expedite the ability of industry to improve the 'value' of academic research. Foresight, no doubt, will be taught as one of the many new master's degrees which, in the government's proposal, are introduced as a more cost-effective alternative to the doctorate of philosophy. This

should have been easy to predict in the light of the closures of university philosophy departments during the past 10–20 years.

I am not suggesting that research in higher education and government institutes should not be subject to change. Indeed, it is crying out for change, particularly in the career structure of research scientists. Accountability, when well founded, can only contribute towards research excellence, but basic science in universities and research institutes should not be made to account for the present slump in the industrial and manufacturing base of the nation. Basic science in Britain is still exceptional and industry and the government must decide how to make the most effective use of this asset and not change the science base to suit its short-term needs. If I were to be the first graduate of the government's School of Foresight and I were to base my dissertation on the White Paper, then I would rightly fear not only for my future as a research scientist but more importantly for the future of basic science in the United Kingdom.

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Human goodness?

SIR — Brian Josephson's concept of religion as an attempt to maximize "human goodness" (*Nature* 362, 583; 1993) is naive and ignores everything we know about religion. The *sine qua non* of any religion is the existence of supernatural forces. On this point the validity of religion stands or falls; genetics is simply irrelevant.

Of the known religions, that of the Aztecs perhaps best illustrates the place of "human goodness" in religion. The Aztecs' religion involved human sacrifice, as did the religions of Central America and Mexico that preceded them. The victim, often a prisoner of war, was spread-eagled on the altar by four priests, while a fifth cut his chest open with an obsidian knife and took out the heart. The victim's head was then severed and put on display in front of the temple while the rest of his body was butchered for meat, to be eaten by the priests or the soldiers who had captured the victim.

This was not a small operation, as Bernal Díaz del Castillo, a *conquistador* who fought with Cortéz, observed: "I remember that in the plaza where their oratories stood, there were piles of human skulls so regularly arranged that one could count them, and I estimated them at more than a hundred thousand. I repeat again

that there were more than one hundred thousand of them. And in another part of the plaza there were so many piles of dead men's thigh bones that one could not count them; there was also a large number of skulls strung between beams of wood, and three priests who had charge of these bones and skulls were guarding them. We had occasion to see many such things as we penetrated into the country for the same custom was observed in all towns, including those of Tlaxcala." (*The Discovery and Conquest of Mexico*, Farrar, Straus and Cudahy, 1956, p. 119).

In the Old World, human sacrifice was also a part of the religion of the Carthaginians, who took it with them wherever they established colonies (A. Moscati, *Scientific American*, February 1975, 80–81). In their case the victims were female children, brought to the temple by their parents to be immolated in the temple oven or outdoor burning ground known as a *tophet*. Had Rome lost the Punic Wars (had Hannibal sacked Rome after Cannae!) theirs would probably have become the established religion of the Ancient World.

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Rustum Roy

SIR — The Penn State chemistry faculty wishes to express its concern about a recent incident involving an attack by Rustum Roy, a professor of the solid state, on Professor Patricia Bianconi, a faculty member of our department. Bianconi published in *Nature*¹ a carefully referenced paper clearly stating the history of this area, which led to her successful demonstration that ordered CdS crystals can be precipitated in an organic polymer network.

Roy felt that some earlier work² from his laboratory should have been cited. Rather than directly contacting Bianconi, he submitted letters to journals and funding agencies. We, as colleagues of Bianconi and as scientists ourselves, were dismayed by this course of action. Moreover, on carefully reading both Bianconi's and Roy's articles, we do not feel she slighted him by not referencing his article.

We are delighted that a public apology has now been offered by Roy³. It is time to close this issue and allow a promising young scientist and colleague to get on with her scholarly work.

Andrew G. Ewing
on behalf of 32 members
of the chemistry faculty,
Pennsylvania State University,
University Park, Pennsylvania 16802, USA

1. *Nature* 349, 315 (1991).
2. *Mater. Lett.* 2, 245 (1984).
3. *Nature* 359, 472 (1992); *Science* 258, 874 (1992).

Lessons in reducing volcano risk

Robert I. Tilling and Peter W. Lipman

Since 1980, volcanologists have confronted more volcanic crises than any time since the Mont Pelée catastrophe in 1902. Good science alone will not do the job of reducing volcano risk.

Vulcanology [is] the Cinderella science which only marches forward on the ashes of catastrophe¹.

SINCE the 1980 eruption of Mount St Helens, volcanoes throughout the world have been repeatedly in the news. The 1980s witnessed more volcanic disasters and crises, as well as eruption-caused fatalities, than any decade since the beginning of the century, when in 1902 three eruptions in a 6-month period killed more than 36,000 people (see table and Fig. 1). With the eruptions at Unzen (Japan) and Mount Pinatubo (Philippines) in 1991, and Mayon (Philippines) in February this year, the 1990s also appear to be off to a disastrous start. The volcanic disasters in 1902 spurred the establishment of volcano observatories and hastened the emergence of volcanology as a multidisciplinary science. Similarly, volcanic disasters since 1980 have enhanced public awareness of eruptive phenomena and associated hazards, and fostered a renaissance in volcanology. More volcanologists are working on active volcanoes than ever before, but the tragic deaths earlier this year of eight of them at Galeras (Colombia) and Guagua Pichincha (Ecuador) are a reminder that this growth can exact a toll. Since 1979, 15 volcanologists have been killed by eruptions "in the line of duty"² — more than in all previous years of the century.

Here we highlight the progress and challenges in reduction of volcanic risk globally, drawing upon recent experience and focusing on volcano monitoring and eruption forecasting as integral components in mitigating volcanic hazards. We emphasize that technological refinements and new discoveries in volcanology alone provide no panacea.

Hazards large and small

Volcanic eruptions in remote, sparsely populated regions rarely cause human or socioeconomic impact, but hazardous processes associated with eruptions or in near-inhabited areas can produce serious problems. Quiescent eruptions of fluid basaltic lava, such as at Kilauea and Mauna Loa (Hawaii) and Mount Etna (Italy), pose little risk to human lives but can damage property and croplands. For example, nearly 30% of Kilauea's flank south of its east-rift zone have been covered by lava flows since 1955, and Kilauea's current east-rift eruption, which began in 1983, has destroyed over 180

structures (Fig. 2) and buried kilometres of highway. It is too early to know if the recently published lava-flow hazard-zone map for Hawaii³ will influence local authorities in making future land-use decisions, but this map already has caught the attention of the cost-conscious insurance industry and lending institutions, and of citizens' groups — pro and con — concerned about geothermal development in areas susceptible to lava-flow hazards. This map was prepared jointly by the Hawaii Office of State Planning and the Hawaiian Volcano Observatory of the US Geological Survey (USGS), representing the first direct collaboration (not in a crisis) between volcanologists and land-use planning officials in delineating and publicizing hazard zones. We view this accomplishment with optimism; prudent land-use planning can reduce volcanic risk in regions not already densely developed, such as Hawaii. We urge government authorities to include evaluation of volcanic hazard-zone maps, along with socio-economic and political factors, in the development of long-range land-use plans. Such options cannot be exercised feasibly in volcanic regions already densely populated, or whose land use has long been fixed by political, traditional or cultural precedents.

Pyroclastic flows and mudflows associated with explosive eruptions at non-basaltic, plate-boundary volcanoes pose much greater hazards than do lava flows. During this century, more than 85% of eruption-related fatalities worldwide were caused by pyroclastic flows and mudflows.

Steeply incised volcanic terrains subject to heavy rainfall, such as Indonesia, the Philippines and central America, are vulnerable to post-eruption hazards (mudflows, floods, secondary pyroclastic flows) generated by rain-induced remobilization and flow of unconsolidated volcanic debris. Since 1991, valleys draining Pinatubo have been devastated repeatedly during the monsoon seasons by such events, which are likely to prevail for years before stable drainage systems are re-established.

In the 1980s, people also became aware of a volcanic hazard unimagined early in the century: encounters between jet aircraft and volcanic ash, as occurred during the eruptions of Galunggung (Indonesia) in 1982, Redoubt (Alaska) in 1989 and Pinatubo in 1991. More than 60 planes, mostly jumbo jets, have been damaged by such encounters, and several planes experienced total power loss, necessitating emergency landings⁴. Luckily, no encounters with ash to date have caused a fatal crash, but thousands of passengers have been at risk and millions of dollars have been spent on repairs or replacement of aircraft parts. Volcanologists, air-traffic control specialists, aircraft manufacturers and the International Civil Aviation Organization have joined forces to respond to this volcano threat, expected to grow as worldwide air travel increases. At the first international symposium on this type of volcano hazard in Seattle, Washington, in July 1991 and subsequent workshops, the participants recognized that, at present, avoiding an ash cloud is the only practical

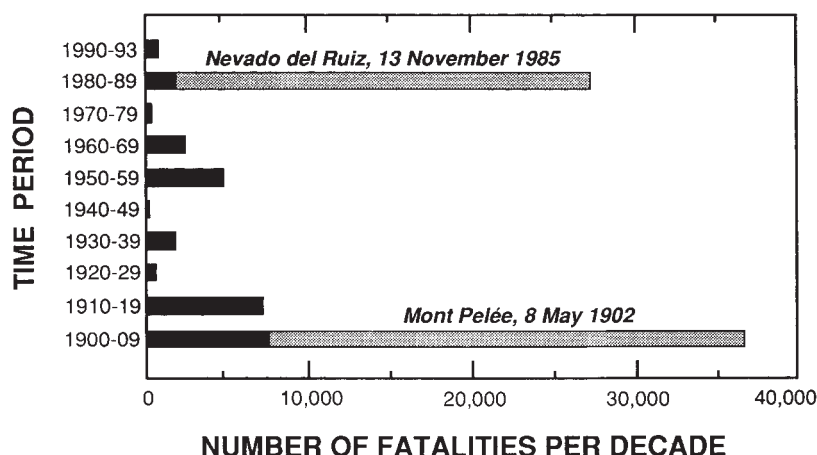


FIG. 1 Volcano-related fatalities by decade for this century until May 1993. Shaded bars, fatalities from the two deadliest events. Modified from Fig. 1 of ref. 17.

MAJOR VOLCANIC DISASTERS AND CALDERA CRISES 1980–93

Volcanic disasters			
Year	Volcano	Fatalities	Remarks
1980	Mount St Helens (United States)	57	Worst volcanic disaster in country's recorded history
1982	El Chichón (Mexico)	>2,000	Worst volcanic disaster in country's record history
1982	Galunggung (Indonesia)	27	Relatively few deaths, but tremendous economic loss and human suffering
1985	Nevado del Ruiz (Colombia)	>25,000	Worst volcanic disaster in country's recorded history
1991	Unzen (Japan)	43	Eruption continuing, 3,000 people still remain evacuated from their homes
1991	Mount Pinatubo (Philippines)	~800*	Largest eruption in country's recorded history; widespread destruction and socio-economic disruption. Given its huge size, fatalities were remarkably few
1993	Mayon (Philippines)	75	Unexpected, relatively small-volume eruptions produced deadly pyroclastic flows; 60,000 people evacuated
Crises at restless calderas†			
1980–84	Long Valley (United States)		Volcanic unrest continuing, but intermittent and less intense than during early 1980s
1982–84	Campi Flegrei (Italy)		Located in heavily populated region near Naples; during height of crisis, volcanic seismicity resulted in collapse of older structures, prompting temporary evacuation of 40,000 people
1983–85	Rabaul (Papua New Guinea)		During height of crisis, officials issued a Stage-2 Alert and were prepared to order evacuations of populace. Crisis abated quickly after issuance of alert

* About 300 deaths during the eruption (mostly from collapse of ash-laden roofs) and 400–500 post-eruption deaths in evacuation camps.

† None of the crises at restless calderas culminated in eruptive activity, but each caused socio-economic impact and serious scientific and public concern.

solution. Immediate efforts are needed to improve: (1) identification and tracking of ash clouds; (2) warning and communication procedures between volcano observatories, air-traffic controllers and pilots; and (3) education of pilots to make them aware of the ash-cloud hazard⁴.

The larger the eruption, the greater the hazard potential. Yet recent experience indicates that the impact, particularly fatalities, is determined principally by factors other than eruption size: proximity and density of population, efficacy of mitigation and luck — good or bad. For example, the 1912 eruption of Novarupta (Katmai, Alaska) — the most voluminous (~ 13 km³ magma) to date in this century — occurred in a remote region and,

accordingly, caused no known fatalities and only minimal property damage. By contrast, the 1985 mudflows at Nevado del Ruiz (Fig. 3) that buried more than 25,000 people (see table) were triggered by a small eruption (~ 0.03 km³ magma); the volume of the phreatic explosion at Galeras in January 1993 (ref. 5) that claimed nine lives was much smaller. Even tiny eruptions can cause big problems.

The greatest, but lowest-frequency, volcano-related hazards are the huge caldera-forming eruptions ($\leq 10^3$ km³ ejecta) from large silicic magmatic systems, such as occurred at Yellowstone during the past two million years. Within recorded history, the world fortunately has been spared calamitous eruptions on

the scale of Yellowstone, but significant unrest at three caldera systems during the 1980s (table) — Long Valley (United States), Campi Flegrei (Italy) and Rabaul (Papua New Guinea) should remind us not to become complacent. Recently recognized as other large-scale but infrequent volcano-related hazards (recurrence interval ~ 10⁴ yr) are prodigious submarine landslides offshore from oceanic volcanic islands, such as Hawaii and Réunion (Indian Ocean). These landslides result from catastrophic failures of mobile flanks of active volcanoes (for example, ref. 6). The discovery of wave deposits ~ 300 m above sea level on the island of Lanai (Hawaii) attests to the enormous tsunamis generated by such volcanic collapses.

The Ruiz volcanic disaster was the worst since the 1902 Mont Pelée eruption (Fig. 1), but it could have been averted. As summarized by Voight (page 349 of ref. 7), the Ruiz "... catastrophe was not caused by technological ineffectiveness or defectiveness, nor by an overwhelming eruption, ... but rather by cumulative human error — by misjudgement, indecision and bureaucratic shortsightedness. Armero could have produced no victims, and therein dwells its immense tragedy." In contrast, considering the large size (> 5 km³ magma) of the 1991 Pinatubo eruption (Fig. 4) and the population density of the surrounding region, the fatalities caused directly by the eruption (~ 300, table) were comparatively few, thanks largely to the swift and effective response by scientists and emergency-management officials. Volcanologists can take pride in the largely successful mitigation at Pinatubo, but we cannot assume that similar successes will be routine in future.

Luck in part determines the impact of an eruption. Had the 1980 eruption at Mount St Helens not been on a Sunday, or occurred an hour later, the fatalities would have been much greater, because more people, including volcanologists, would have been at work within the restricted-access zone around the volcano. Similarly, had the ill-fated field excursion to Galeras Volcano not been rescheduled because of a planned power cut for the city of Pasto⁵, in all probability no one would have been close enough to be injured by the relatively feeble phreatic explosion.

Can we predict eruptions?

For densely populated volcanic regions, an improved predictive capability is the only practical means to reduce volcanic risk, thus placing responsibility squarely on volcanologists to warn of impending activity. As with hazard assessments, long-term eruption forecasts are based on knowledge of a volcano's eruptive style and history, which can be deciphered only from geological, geochemical and dating

studies of volcanic deposits (see ref. 8). Data from these basic studies allow determination of the distribution, nature and recurrence interval of previous eruptions and attendant hazardous processes. To be useful in reducing volcanic risk, such information should be in hand before a volcanic crisis strikes and cannot be obtained overnight — a seemingly obvious fact that is commonly ignored or overlooked by funding agencies and, regrettably, even by a few volcanologists.

Short-term eruption forecasts rely primarily on volcano monitoring — the systematic observation and measurement of a volcano's visible and subsurface activity, using geological, geophysical, geodetic and geochemical techniques. During the past 20 years, volcano-monitoring techniques, particularly geophysical methods, have been refined considerably, reflecting advances in electronics and computerized data acquisition and processing. Perhaps the most useful innovation for complementing permanent seismic networks, or for quick deployment at previously unmonitored volcanoes during a crisis, is the development of personal computer (PC)-based seismic-data acquisition and analysis systems⁹, "realtime seismic amplitude measurement" (RSAM)¹⁰ and realtime "seismic spectral amplitude monitor" (SSAM)¹¹ and associated supporting software. These systems, which are portable and cost-effective, can serve as the core of 'mobile observatories' and have been useful at Mount St Helens, Redoubt and Pinatubo.

Satellite-based techniques (for example, ref. 12) represent another significant advance in volcano monitoring. For example, the Total Ozone Mapping Spectro-

FIG. 3 Aerial view of the remains of the city of Armero, Colombia, buried by massive mudflows triggered by a small eruption of Nevado del Ruiz on 13 November 1985.

meter (TOMS), in addition to monitoring ozone levels from space, can measure SO₂ output from eruptions, enabling the detection and tracking of volcanic plumes. Also encouraging are geodetic applications of the Global Positioning System (GPS), which is approaching the few-parts-per-million resolution needed for diagnostic volcano monitoring. GPS-based geodesy, particularly as equipment costs and data-processing time are reduced, promises an all-weather technique that can augment and/or replace conventional geodetic volcano monitoring.

Still, with the recent advances in volcano monitoring, can we predict eruptions? The answer is rarely, if we rigorously

define 'prediction' as a precise statement "... of the time, place, and ideally, the nature and size of impending activity" (p. 397 of ref. 13). Since 1980, the near-perfect record of predictions of dome-building activity at Mount St Helens, and the accurate predictions of eruptions at Sakurajima Volcano (Japan), are among the few documented successes. However, these eruptions were small, occurred at volcanoes with 'open' vents, and posed hazards only to those working near the active vent areas. The continuing eruption and volcanic crisis at Unzen re-emphasizes the sobering reality that, even with state-of-the-art monitoring and data analysis practiced by our Japanese colleagues, it may not be possible to predict renewed 'vent-opening' activity at volcanoes that erupt infrequently. At present, no volcano-monitoring method can reliably predict large explosive eruptions.

Problems and opportunities

By the year 2000, the population at risk from volcano hazards is likely to increase to at least 500 million, comparable to the estimated world population at the beginning of the seventeenth century. Therefore, volcanologists face a formidable challenge in providing reliable and timely warnings of eruptions. Below we outline several paramount issues.

Need for additional, reliable, real-time monitoring systems. At present, seismic networks provide the only commonly used, and most reliable, realtime means to monitor volcanoes. Data from continuously recording tiltmeters and other geodetic instruments are too few and, in some cases, insufficiently reliable. Experience shows that optimum monitoring is achieved by a combination of methods,

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

FIG. 2 Wahaula Visitor Center, Hawaii Volcanoes National Park, was one of more than 180 structures overrun by lava flows (foreground) from the 1983-present eruption at Kilauea Volcano.

E. W. Wolfe/USGS



FIG. 4 Aerial view towards the northeast, showing the Marella Valley choked with pyroclastic-flow deposits from the 15 June 1991 eruption of Mount Pinatubo (upper right). These valley-filling deposits of unconsolidated debris greatly altered the drainage patterns in the area, and their remobilization and erosion generated destructive post-eruption mudflows with every heavy rainfall during subsequent monsoon seasons.

rather than reliance on one. We must develop rugged, reliable realtime systems to measure vertical and horizontal ground displacements, non-seismic geophysical parameters (such as gravity and electromagnetic fields), and the composition and flux of the principal volcanic gases (such as SO_2 , CO_2). Towards this end, the USGS has developed prototype telemetered GPS and gas-sensing systems.

New approaches to eruption prediction.

To date, volcanologists have relied almost entirely on pattern recognition in monitoring data and past eruptive behaviour to forecast the place, time, type and magnitude of possible gravity. Although this empirical approach has worked at a few well-studied, frequently active volcanoes, it has limited general application. To complement and quantify the pattern-recognition approaches, it is critical to understand better the source mechanisms producing the precursory patterns observed. Particularly promising is recent research on long-period volcanic seismicity (for example, ref. 14) that almost invariably precedes eruptive activity.

Study of more volcanoes. Breakthroughs in predictive capability have usually occurred at the few volcanoes already intensively monitored, mainly in the developed countries. Many of the highest-risk volcanoes are elsewhere, however, and most of these are inadequately monitored, if at all. Thus, we expect the greatest advances in mitigation of hazards in the near future to come from applying existing technology to these volcanoes, rather than from technological refinements or new scientific discoveries alone.

More effective interaction with civil authorities and the public. Monitoring data or eruption forecasts, no matter how timely or precise, will not reduce volcanic risk unless they can be communicated effectively to, and acted on, by

emergency-management authorities. Volcanologists must establish more effective communication with government officials, news media and the affected populace lest they lose any clout that they might have in influencing the decisions made by civil authorities during a volcanic crisis.

More effective international cooperation.

Most high-risk volcanoes are in densely populated developing regions, which lack sufficient human and economic resources for adequate study. Ideally, countries in these regions, individually or collectively, would become self-sufficient in volcanology and related emergency-management infrastructure. Such institution building may require generations. In the interim, scientists in developed countries must provide mutual assistance — through cooperative bilateral or international programmes. Some notable successes have been achieved recently by the rapid deployment of 'scientific response' teams, some multinational, to volcanic trouble spots. For example, the prompt dispatch to Mount Pinatubo of the USGS Volcanic Crisis Assistance Team, in coordination with the Volcano Disaster Assistance Program (Office of Foreign Disaster Assistance, US Department of State), to work with colleagues of the Philippine Institute of Volcanology and Seismology was important in the overall, generally successful hazard mitigation.

Another cooperative international effort is the Decade Volcano Demonstration Project, a contribution of the International Association of Volcanology and Chemistry of the Earth's Interior (IAVCEI) to the UN-sponsored International Decade for Natural Disaster Reduction (IDNDR) for the 1990s. The Decade Volcanoes project aims to focus "intensive, integrated, multidisciplinary, multinational cooperative demonstration work on the entire range of activities needed in

volcanic hazards mitigation" at selected high-risk volcanoes throughout the world (p. 88 of ref. 15; bold emphasis therein).

We are disappointed by the slow progress made to date on the Decade Volcanoes project and by the lack of tangible plans for future activities. To realize the full potential of this promising project during the IDNDR, money as well as moral support is needed from the United Nations and other sources, and soon; a third of the IDNDR has already elapsed.

Finally, as more active or potentially active volcanoes are studied, more volcanologists are exposed to personal risk, as recent events at Unzen, Galeras and Guagua Pichincha attest. We are greatly saddened by the loss of colleagues while making on-site studies. Much volcano-monitoring data can be collected instrumentally and telemetered to a central facility for analysis. However, instruments must be installed and maintained in the field, and many other observations and measurements can be made only on-site; thus fieldwork inevitably entails temporary exposure to hazardous working conditions. The occupational hazard of volcanologists is somewhat akin to that of police and firefighters; imposition of overly stringent safety standards could preclude meaningful observation on or near potentially dangerous volcanoes. Volcanologists must be willing to assume some personal risk, but they also bear the responsibility for taking reasonable safety precautions, so as not to endanger their own lives or the lives of others in the field party. We concur with the official IAVCEI position¹⁶: "The work of volcanologists never will be without risk, but careful assessment of the value of data, insight, and experience that might be gained versus associated risk could reduce the loss of life in our profession." □

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Wilful public misunderstanding of genetics

This week's excitement over a report suggesting a genetic basis for homosexuality has been overdone, suggesting that there is a long way to go before public understanding is enlightened.

So after all, there is a gene for male homosexuality! That seems to have been the common reaction to last week's announcement of a genetic linkage between male homosexual behaviour and a polymorphism of the X-chromosome. "We knew it all along," the homophobes have been telling dinner-party audiences. "Those guys (or gays) are *different* from you and me."

The responses of gays themselves have been variable. Some have welcomed the idea that behavioural characteristics with which they are personally content may have been given the status of being inheritable, as are the colour of hair or skin, so that they may claim immunity from the discrimination and ostracism to which gays have been exposed. Others are alarmed that the discovery of a genetic basis for their sexual orientation may quickly be followed by an attempt to get rid of it, perhaps by amniocentesis and abortion, from the population. Absurdly, a member of the British Parliament has even demanded legislation to prevent the use of this knowledge further to discriminate against the gay population. But the general complacency is entirely misplaced, as are the hopes and fears of gays themselves.

This wave of excitement has been generated by a report of a genetic study by Dean H. Hamer and colleagues at the National Cancer Institute. A measured assessment of the research appears on page 288 of this issue. In brief, the DNA of 33 out of 40 pairs of brothers both declaring themselves to be exclusively homosexual in orientation was investigated with a series of previously defined genetic markers spanning the whole length of the X-chromosome.

The upshot is a significant correlation between declared homosexuality and the inheritance of a genetic marker near the end of the long arm of the X-chromosome. Thanks to the high density of markers on the X-chromosome, if there is a gene, it will probably lie within 4 million base-pairs or so of an identifiable marker. That may be a small fraction of the whole human genome, but it is still a huge stretch of DNA to analyse nucleotide by nucleotide.

So is there a gene for homosexuality hidden in that DNA? Properly, the authors of the study raise the question and then properly enter a formidable list of caveats. For example, they emphasise the need for confirmation of their study, evidently conscious of how previous claims of a genetic basis of behavioural traits in people (manic-depressive illness among

Amish people and schizophrenia) proved false. One obvious pitfall in this business is that of building into the choice of a sample of subjects, in this case the pairs of brothers, a bias of some kind. The authors have been careful, but who can tell how careful is careful enough.

It is also possible that the interpretation may be falsified by extraneous influences. What if it is accepted, for example, that it is true what the psychoanalysts say that male homosexuality is in part determined by the influence of an over-loving mother? And what if the gene located at the end of the X-chromosome does not determine male homosexuality, but instead plays a part in telling whether a mother is "over-loving" in the appropriate sense? Then the gene concerned would be strictly irrelevant to the causation of male homosexuality, whose determinants would remain those of nurture rather than nature.

That is not to rubbish an important finding; either way, a genetic determinant of a behavioural trait would have been identified. But it is entirely possible that further investigation will show that the familial incidence of traits for sexual preference is not at all genetically determinate. The data now published would be more informative on that score if they said more about the incidence of the inheritance of the X-linked markers in the general population.

The authors suggest that the frequency of the putative allele linked with male homosexuality is roughly 1 in 50, but that estimate strictly applies only to their sample of brother pairs and the relatives thereof who have volunteered DNA. It could easily be that the frequency of the allele in the general population is much greater, in which case the link between its inheritance and homosexuality will be weakened. Time (and analysis by independent methods) will answer that.

But it is also plain, as the authors say as clearly as they can, that their data do not explain the homosexuality of the seven (out of 40) brother-pairs in whom the putative allele cannot have been inherited in the fashion described. Is their behaviour determined by other genes, or is nurture solely responsible? Just as if somebody had found a gene linked with, say, the inheritable part of IQ, it would be realised that little had been done to improve the education of the young, so the recognition that some part of the spectrum of male homosexuality is genetically determined (which is not certain) does not of itself simplify

the conduct of family life.

That is why it is disconcerting that the treatment by the general press in Britain of what may be an important step forward in the genetics of human behaviour has been so patchy. Most have approached the problem with solemnity, eager to display the responsibility with which a delicate but important issue is being tackled. But only a few have succeeded.

Among the serious British press (the tabloids are something else), for example, the *Sunday Telegraph* filled a whole page last weekend with three articles with headlines **Born to be gay** (reactions from male homosexuals), **A lot of mothers are going to feel guilty** (subtitled "...scepticism mingled with thoughts of Hitler") and **The gene genie comes out fighting**. The same newspaper carried a leading article suggesting "an extraordinary alliance of gay rights activists and the Right to Life lobby" in defence of the wild-type human genome.

Another, the Sunday version of the *Independent*, which gave a page-full factual account of what the new development consists of and what it implies, thought it worthwhile complaining in a leading article, under the heading of **The genetic tyranny**, that the governments "contributing a total of \$2 billion to an attempt to map the entire human genome" have not "given a second's thought" to preventing the misuse of the data that may be gathered.

The worry in all this is neither ethical nor educational, but the tendency of even sober-sided newspapers to overdramatise discoveries only, afterwards, to complain that they have been misled. Even a casual reading of the original article will reveal a commendable list of caveats. Every serious person telephoned by the newspapers has repeated them and others (and often has been reported as having done so). Yet the overall effect is to pass off inference as fact, and to conceal the certainty that if there is a genetic component of male homosexuality, its influence will be much more complicated than the simple picture rehearsed in the past few days.

In Britain, Mr William Waldegrave the science minister says he plans to spend £100,000 on the encouragement of public understanding of science, a cause long since taken up by the Royal Society. Perhaps the most urgent need is for ways of telling a good tale about developments where the story-line is neither black nor white.

John Maddox

Genes make worms behave

Robert K. Herman

TWENTY years ago Sydney Brenner described¹ an electrode-less plan for attacking the problems of neural development and physiology in the small nematode *Caenorhabditis elegans*. He proposed to set the groundwork by reconstructing the entire nervous system of the worm by serial section electron microscopy. Given the resulting wiring diagram, he thought it might be possible to make guesses as to how the nervous system worked. A second aspect of his plan was genetics:

assignments were made in two ways. First, killing a particular subset of GABAergic neurons by a laser microbeam resulted in a specific behavioural abnormality. And second, mutants defective in GABA biosynthesis exhibited all three behavioural abnormalities.

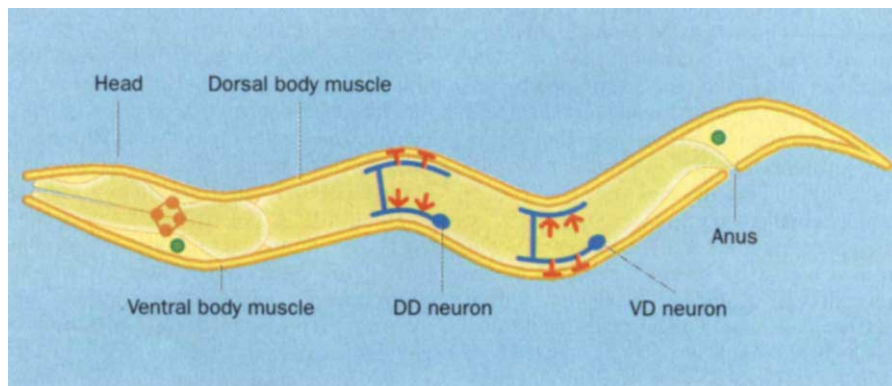
From the known connectivities of the subset of GABAergic neurons responsible for a specific behaviour, it was possible in each case to propose a model for how the neurons exert their effects. Actually,

thought to be inhibitory, in this case to dorsal or ventral muscles in the head. But McIntire *et al.* provide good evidence that the GABAergic neurons that affect defecation are excitatory; this is a surprising result because GABA is generally thought to be inhibitory.

In the other paper, McIntire *et al.*² analyse uncoordinated mutants that shrink. The mutations were in five genes, all identified by Brenner. The *unc-25* gene seems to encode the GABA biosynthetic enzyme. Mutations in *unc-30* affect the differentiation of only those 19 GABAergic neurons that are involved in dorsoventral waves of contraction; all other neurons and behaviours seem normal. Two other genes, *unc-46* and *unc-47*, were implicated in GABA release, and *unc-49* was implicated, by a pharmacological assay, in the body muscles' response to GABA. With a function for each of these genes identified, cloning and sequencing will surely follow; it would not be surprising if both familiar and hitherto unknown gene products were found.

The techniques illustrated here — killing specific neurons with a laser, studying wiring diagrams, and analysing mutants with specific behavioural abnormalities — are being applied to other neurons and other behaviours in *C. elegans*. As already noted, some non-GABAergic neurons are necessary for coordinated locomotion. Other neurons have been shown to be involved in mechanosensation, chemosensation, egg laying or eating⁷.

Many mutations that alter these behaviours have been identified. Much remains to be done, but it is already clear that some genes, the shrinker *unc* genes being examples, affect specific behaviours by virtue of affecting the differentiation or function of only one or a few classes of neurons. Other genes have a more general role in neuronal function, altering, for example, process outgrowth or guidance, synapse formation, or synaptic vesicle formation or release⁷⁻¹⁰. Most mutants with general neuronal defects that have been studied so far retain some residual neuronal function and lead fertile but uncoordinated lives. Mutants with no neuronal function at all seem to manage embryogenesis but do not grow or repro-



Genes, GABA and behaviour in *C. elegans*. The 19 GABAergic neurons that regulate body muscle contractions are of two types, DD and VD, and receive input (arrows) on either the ventral or dorsal side from motor neurons (not shown) that excite body muscle contraction on the same side; the GABAergic neurons then make inhibitory output (crossbars) to relax muscles on the opposite side and thereby promote dorsoventral bending. Four GABAergic neurons in a ring in the head prevent the head from bobbing too far from side to side during foraging. Two GABAergic neurons (cell bodies in green, one in head and one in tail) control contraction of enteric muscles near the anus for defecation; these cells have long processes that are not illustrated.

single-gene mutants exhibiting aberrant behaviour, such as uncoordinated movement, were to be analysed to address the question of how genes specify development and function of the nervous system. In two papers beginning on page 334 of this issue, McIntire *et al.*^{2,3} demonstrate that work on Brenner's plan, with a few tricks added over the years, is progressing very nicely.

The structure and connectivity of the complete nervous system of the *C. elegans* hermaphrodite, which has 302 neurons, were described in 1986 (ref. 4). McIntire *et al.*³ now show that 26 of the 302 neurons express the neurotransmitter GABA and that 25 of these are involved in three distinct behaviours (see figure). Nineteen GABAergic neurons help regulate the waves of muscle contraction that propagate along the body of the worm and propel it forward or backward. Four other neurons are involved in limiting the extent of the back-and-forth sweep of the head that occurs during foraging for food, and two neurons direct the final (expulsive) step in defecation. (No function was found for the 26th neuron). The behavioural

in the case of the waves of contractions along the body, the work of McIntire *et al.* strengthens a model that had been proposed previously⁵, on the basis of the neuronal wiring and earlier laser killing experiments in *C. elegans*, and electrophysiological and biochemical work carried out on the much larger nematode *Ascaris*⁶.

The worm's body bends are caused by phases of contraction and relaxation of dorsal and ventral muscles; excitatory motor neurons (different sets specifying forward and backward locomotion) activate the muscle contractions and also activate the GABAergic motor neurons, which inhibit muscle contraction on the opposite side of the animal. The GABAergic neurons thus help to keep the dorsal and ventral muscle contractions out of phase and thereby promote dorsoventral flexures. If the GABAergic neurons are nonfunctional, dorsal and ventral contractions can occur simultaneously, causing the animal to shrink in place. The four GABAergic neurons in the head that prevent exaggerated sweeps of the nose during foraging are also

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duce; these mutants are more difficult to analyse, but they are likely to become a rich resource for future work.

The most dramatic development in Brenner's plan over the past 20 years has been in the cloning of genes defined by behavioural mutants. As the work of McIntire *et al.* illustrates, however, the powerful molecular technologies have not lessened the importance of the whole-

animal approach. It is whole animals that exhibit behaviours, and the best test of the significance of particular cells or genes in specifying a behaviour involves inactivating the cells or genes in a living animal. □

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GALACTIC STRUCTURE

More whirls in the whirlpool

Jeffrey Kenney

THE reputation of the celebrated Whirlpool galaxy, the most famous of all grand-design spiral galaxies, has just been given another boost. Its remarkable inner spiral arms, discovered in near-infrared pictures by Zaritsky and co-workers¹ and described on page 313 of this issue, extend much closer to the centre of the galaxy than had previously been realized. Three continuous revolutions can be traced, twice as many as in any previously known spiral arm. The extent and continuity of the spiral arms are much more than a detail or a new record. They pose a strong challenge to our theoretical understanding of grand-design spiral structure in galaxies.

Astronomers recognize two types of spiral structure in galaxies: 'flocculent' and 'density-wave'. Flocculent (or chaotic) spiral galaxies contain a multitude of short spiral-arm fragments, none of which can be traced for more than a small fraction of a full revolution. Flocculent spiral arms are similar to the whirls created by pouring cream into a cup of stirred coffee — both are material structures which are sheared by differential rotation into a spiral shape. It is star-forming clouds of gas and dust, not the old stars, that form spiral structures in flocculent galaxies. NGC4736 and NGC2841 are two good examples of flocculent-arm galaxies from the Hubble atlas².

Dramatic and beautiful, density-wave spiral arms are fundamentally different. In contrast to flocculent arms, density-wave arms are arms in the stars as well as the gas, and they can be traced for at least half a revolution. Grand-design spirals such as the Whirlpool (catalogued as M51) are the most visually impressive of the density-wave galaxies because they have a simple two-fold symmetry. The spiral structure in other density-wave galaxies appears less regular because it is probably a superposition of two-arm, three-arm and perhaps other patterns³. Density waves force orbiting stars and gas to slow down in the arms, and the resulting traffic jams create spiral-shaped regions of enhanced density. Shear due to differential

rotation gives the density wave its spiral shape.

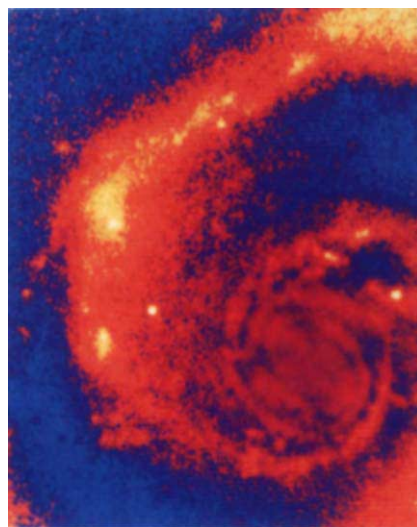
It is still debated whether the waves are more like transient ocean waves that pass through molecules of water, or more like standing waves of sound on the surface of a beaten drum. Although theoreticians have postulated that density waves might be created by an internal instability, observations have shown that virtually every known grand-design spiral galaxy has a central bar or a nearby galaxy whose tidal forces probably triggered the waves.

Analytical results on density waves can be obtained only for the most simplified systems, so numerical simulations are an important way to study spiral structure in more complex and realistic galaxies. Simulations show that tidal forces from the nearby companion galaxy NGC5195 are able to create the outer spiral arms of M51, but are too weak to trigger arms in the inner galaxy directly. Density waves propagate in towards the middle of the galaxy only if the self-gravity of the stars and gas are included in the simulations, so

it is the outer arms that trigger the inner arms.

But no one has yet seen tidally generated arms in simulations which wind for three full revolutions or extend as close to the centre as do the arms of M51 (see figure). The underlying idea that grand-design arms are density waves is almost certainly still correct, but the precise physical recipe that predicts their behaviour continues to elude us. At this point we can't be sure whether the physical model is missing an important ingredient or whether there is some unforeseen interaction between the ingredients. Alternatively, it may simply be that simulations with the same physics but with greater numerical resolution are needed to see the small-scale structures revealed in the near-infrared images.

Theory must explain how a coherent wave generated by a companion galaxy located at 12 kiloparsecs has penetrated to within 400 parsecs of the nucleus. The behaviour of a density wave is complex because the disks of galaxies through which density waves propagate are heterogeneous mixtures of gas and stars, and waves act rather differently in gas than in stars. A wave propagating in towards the centre of the galaxy through stars will be absorbed at a special location in the disk referred to as the inner Lindblad resonance, and this limits the inward radial extent of a stellar density wave and prevents a standing wave pattern from forming. But a wave propagating through gas can pass through this resonance, enabling a gaseous density wave to extend further inwards than a stellar density wave. If the gas makes up a significant fraction of the total mass, which it does in the central region of M51, then the gravitational force of the gaseous arms will attract the stars



Near-infrared image (left) of the Whirlpool galaxy, obtained by Zaritsky *et al.*¹ (central part of galaxy only); and optical image (right) of M51 and its companion NGC5195, obtained with the 4-metre telescope of Kitt Peak Observatory, showing the outer spiral arms.

and may allow a spiral pattern to continue through the inner Lindblad resonance.

Unfortunately, it is generally difficult to determine from observations where the resonances in galaxies are located or even if they exist. M51 is not in a steady state because of its collision with NGC5195, and there may be no normal Lindblad resonances in such a dynamically evolving galaxy. Perhaps the lack of a true resonance in M51 allows the density wave to penetrate deep into the galaxy core. Regardless of the explanation, the observations demonstrate that the disk of a galaxy can be a surprisingly good waveguide.

Density-wave spiral arms will become more tightly wound as the galaxy rotates differentially, and will wind up completely within several rotation periods after their creation unless they are constantly regenerated. Consequently it is easier to understand transient spiral density waves than persistent ones. We are viewing M51 at a special time in its history, in the midst of a collision with NGC5195. The optical photograph shows that much of M51's outer spiral structure is related to the collision, so the tightly wound inner arms may indeed be transient waves which are now in the process of being wound up. The ultimate fate of these arms may be to merge and form a ring.

The remarkable inner spiral structure discovered by Zaritsky *et al.* had not been detected previously in M51 or in any other galaxy because it is only observable in the near-infrared ($\sim 1\text{--}5\ \mu\text{m}$) and not at optical wavelengths ($\sim 0.4\text{--}0.7\ \mu\text{m}$). Most of the near-infrared light arises from older giant stars, which are good tracers of the total mass in stars. These giant stars and presumably most of the other stars are organized into the spiral arms seen in the near-infrared picture. At visible wavelengths these spiral arms are obscured by dust and confused by the bright light from young, short-lived stars which have a more irregular distribution. These two problems can be particularly severe in the central regions of galaxies where there is a lot of dust and star formation. The intensity from the inner arms is weak, so that one needs to subtract the smooth component of the starlight to see them clearly. In contrast to the outer

arms, in which the peak stellar density in the arms is about twice that in the interarm region, the inner arms are only slightly brighter than the background. Consequently, the strong outer arms are clearly visible on a simple optical photograph, but the weak inner arms are evident only from a specially processed infrared image.

Although astronomers have been taking optical images of galaxies for more than a century, obtaining high-resolution pictures at near-infrared wavelengths is a relatively new venture, made possible by technological improvements. In the past few years, near-infrared images have revealed previously unsuspected bars^{4,7}, distinctly different spiral structure⁸, hidden nuclei in galaxies⁹ and clearer views of the nucleus¹⁰ and large-scale structure¹¹ of our own Milky Way. Exciting discoveries are bound to continue.

TECTONICS

Making composite continents

William R. Dickinson

EURASIA is the largest modern continent, and its immense size reflects its composite character. Over the past half-billion years, about a dozen individual continental blocks have been welded together along the sinuous lines of modern and ancient mountain belts that now interlace the interior of Eurasia. On page 299 of this issue¹, Şengör, Natal'in and Burtman disentangle the complex tectonic history of the continental heartland of central Asia, and find that the initial stages of its assembly involved more than mere shuffling of mini-continents — huge areas of new continental crust were added to the landmass.

Şengör and colleagues focus on the Altaid orogenic system (or orogenic "collage" in their words — orogeny is the process of mountain building), which evolved over a span of 350 million years between the end of the Precambrian (Vendian) period and the beginning of Mesozoic time (600–250 million years ago). The Altaid realm is a vast arena extending some 5,000 kilometres from east to west through the various nations and provinces of the Sino-Siberian borderlands (see figure).

The events that formed the Altaids pre-dated the Tethyside orogenic belts, shown in the figure, along which continental masses forming the southern and eastern fringes of Eurasia were attached to the Eurasian heartland during Mesozoic and Cenozoic times (250 million years ago until the present). Tethyside orogens^{2,3} are essentially crustal sutures along which drifting continental blocks were joined by the closure of various Tethyan seaways. Tethys, the sister and consort of Okeanos

There is more at stake here than understanding the dynamics of spiral arms themselves. Spiral arms help to drive the evolution of their host galaxies. Density waves trigger star formation in the gas and plump up galaxy disks by increasing the random motions of older stars. Some of the most spectacular activity in galaxies, nuclear starbursts and active galactic nuclei, is probably fuelled by inward radial flows of gas. Spiral arms are one of several mechanisms that transfer angular momentum and cause mass to be driven radially, so their effect on galaxies can be dramatic. It is the beauty of spiral arms that draws our attention, but it is their power that deserves our thoughts. □

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(Oceanus) in Greek mythology, is the name given to the oceanic realm that formerly lay south of Eurasia. Remnants of ancient crust are common among the Tethyside orogens but the Altaids are apparently different.

To decipher the Altaid puzzle, Şengör and colleagues trace the magmatic fronts of former island arcs to establish the trends of successive orogenic belts active during Altaid evolution. In plate tectonic parlance, a 'magmatic front' is the oceanward limit (normally sharp) of subduction-related volcanism and associated plutonism forming a curvilinear belt of arc magmatism. The arc magmatism is caused by descent of oceanic lithosphere (sea floor) into the mantle at an associated oceanic trench or subduction zone lying parallel to the magmatic arc.

By tracing Altaid magmatic fronts from outcropping rocks, the authors conclude that multiple segments of Altaid orogens, now separately identifiable and seemingly independent of one another, are in reality disrupted and displaced fragments of an integrated subduction system they term the "Kipchak arc". The palaeogeography of this complex arc system may have resembled the modern-day Indonesian–Philippine region⁴ in both size and general configuration.

Altaid continental crust is evidently composed mainly of accretionary subduction complexes studded from below with Palaeozoic plutons (cylindrical masses of plutonic rock) and partly covered by derivative volcanics. Accretion occurred by scraping voluminous sediment off the floors of remnant ocean basins⁵, and a good proportion of the igneous magmas

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RÉSUMÉ

Broken hearts

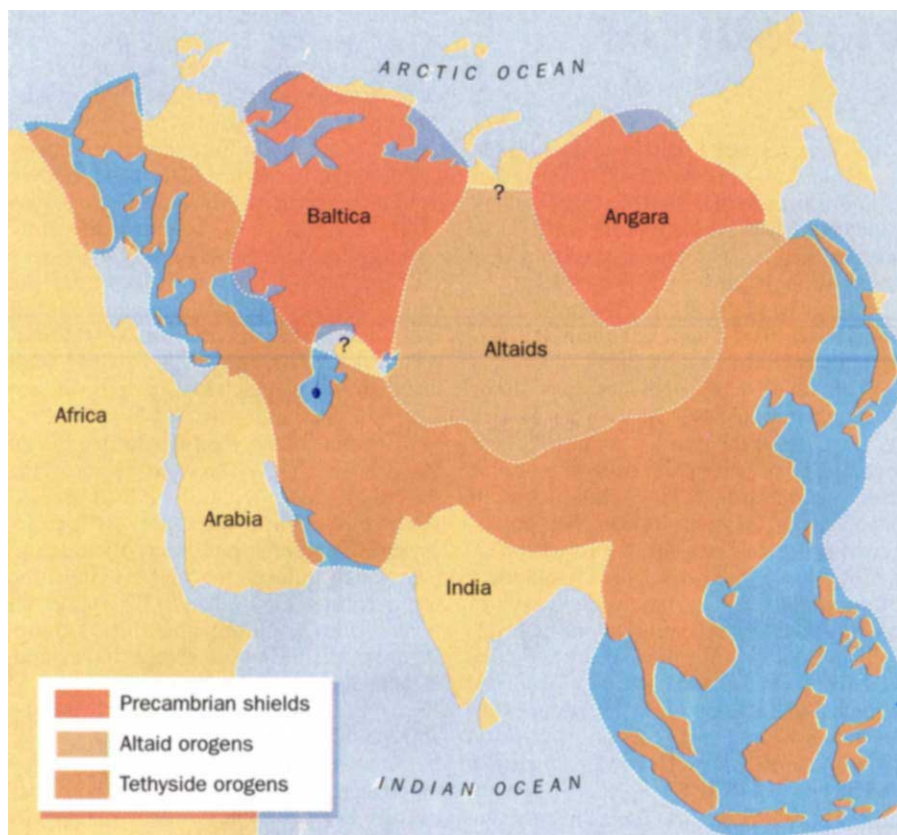
Writing in *The Plant Cell* (5, 621–630; 1993), Chun-ming Liu and co-workers describe work using their new technique of growing very early plant embryos *in vitro*. What they have done is to disturb the development of bilateral symmetry in embryos of Indian mustard by inhibiting auxin transport; unlike other plant hormones, auxin is transported in a directional fashion within the growing plant. Treatment of embryos at the early 'globule' stage with inhibitors of auxin transport prevented formation of the typical heart shape, with two proto-cotyledons, which marks the onset of bilateral symmetry. Instead, the embryo formed a funnel with a collar of cotyledon surrounding the central depression. Surprisingly, these misshapen embryos went on to grow normally, a tribute to the regenerative power of plants.

Nanoballbearings

The crystalline form of the fullerene C_{60} shows a rare combination of properties. It has great mechanical resilience and chemical stability yet is as soft as graphite — so why not use it as a solid lubricant, thought B. Bhushan and colleagues. Accordingly, they prepared thin films of C_{60} deposited on a silicon wafer under high vacuum, then slid the films against a steel ball and calculated the coefficient of friction (*Appl. Phys. Lett.* 62, 3253–3255; 1993). Under optimum conditions, at a temperature of 110 °C, they found that the films outperformed the commonly used solid lubricant molybdenum disulphide, showing lower friction and low wear. Microscopy revealed the formation of nearly perfect spherical wear particles which, it is thought, roll around between the surfaces during sliding. Nanoballbearings for nanotechnology, perhaps?

Out of Africa

In Ghana asthma and other disturbances of smooth muscle are treated with a medicinal herb, *Desmodium adscendens*. O. B. McManus and co-workers (*Biochemistry* 32, 6128–6133; 1993) have looked at the effect of substances isolated from this plant on calcium-activated potassium channels (the so-called maxi-K channels), which are implicated in relaxation of smooth muscle in the airways and in controlling the release of bronchoconstricting agents from the neurons. The most active of three substances (all of which are triterpene glycosides) that McManus and co-workers have characterized inhibits binding of a scorpion toxin to the maxi-K channel, and also reversibly opens the channel. Presumably this relaxes the smooth muscle *in vivo* — which should be good for asthmatics and no doubt also for physiologists.



Location of Altaid orogenic collage in central Asia; Angara is the Precambrian nucleus of Siberia and Baltica is the nucleus of Europe.

represented juvenile material that emerged from the mantle during Palaeozoic time. Exposures of gneissic Precambrian crust are largely absent.

Consequently, Altaid tectonic history is not a tale of abrupt, episodic deformation caused by jostling of pre-existing crustal blocks, but one of a continuous process of migratory orogeny as accretionary subduction complexes were progressively assembled and arc magmatism was subsequently superimposed. Ophiolites (remnants of oceanic crust) within the Altaid collage occur in haphazard patterns dictated by their incremental addition as accreted slivers to growing subduction complexes, rather than as continuous belts along sutures between pre-existing mini-continents.

These observations tell us much about the way the crust could have evolved over at least three billion years before the formation of Eurasia. For some time after plate tectonics became the accepted way of interpreting Phanerozoic global tectonics, many doubted its applicability to the Precambrian period. But this is now widely accepted⁶, and complex patterns of crustal belts young and old within Precambrian terranes are interpreted as recording the assembly of ancient composite continents like modern Eurasia⁷.

In fact, the overall geotectonic theme of Earth history is now seen as the aggregation and fragmentation of composite

supercontinents. Whether the tempo of assembly and dispersal is episodic and essentially random, or truly cyclic and systematic on a global scale, is a question yet to be resolved.

In either case, the lesson of the Altaids is clear. The formation of composite continents can involve more than just the shuttling of pre-existing crustal blocks around the globe by continental drift. If the interpretations of Şengör and colleagues are broadly correct, Altaid evolution provides an example of nascent continental crust generated at sites destined to form the core of a composite continent. Continental drift alone merely rearranges the crustal cards on the global table, but the Altaid example shows how new cards can be drawn from the mantle deck in the process of making a composite continent.

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Pressing for better results

Paolo G. Radaelli and James D. Jorgensen

ON page 315 of this issue, Hiroi and his co-workers¹ present what must be the simplest copper oxide superconductors so far. With only two metallic elements, strontium and copper, superconducting transition temperatures (T_c) of 70 and 100 kelvin are achieved, depending on the crystal structure.

The compounds are members of a homologous series with the general formula $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$. The $n = 1$ compound, $\text{Sr}_2\text{CuO}_{3.1}$, with $T_c = 70$ K, has the well known La_2CuO_4 structure, but with a large number of oxygen vacancies in the SrO_2 layer (see figure). As Sr^{2+} rather than La^{3+} is the cation in this layer, oxygen vacancies are needed to give the correct doping level. The $n = 2$ compound, $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$, with $T_c = 100$ K, has a structure like that of $\text{La}_2\text{SrCu}_2\text{O}_6$ (see figure) but again with oxygen vacancies in the SrO_2 layers. Hiroi and colleagues characterized the structures of the new phases with a combination of X-ray and electron diffraction, and high-resolution lattice imaging in the electron microscope. Higher members of the series ($n \geq 3$) were also seen.

The key to the discovery of these compounds is high-pressure synthesis. When made at ambient pressure, Sr_2CuO_3 has an orthorhombic structure with CuO chains and is not superconducting. But when a small amount of additional oxygen

($\delta \approx 0.1$ atoms per formula unit) is made available under pressures of 6 GPa and at temperatures of 800–900 °C, as Hiroi and colleagues show, the structure converts to one in which the square-planar CuO_2 layers thought to be required for superconductivity are present. The $n \geq 2$ compounds are made under the same conditions by including additional CuO .

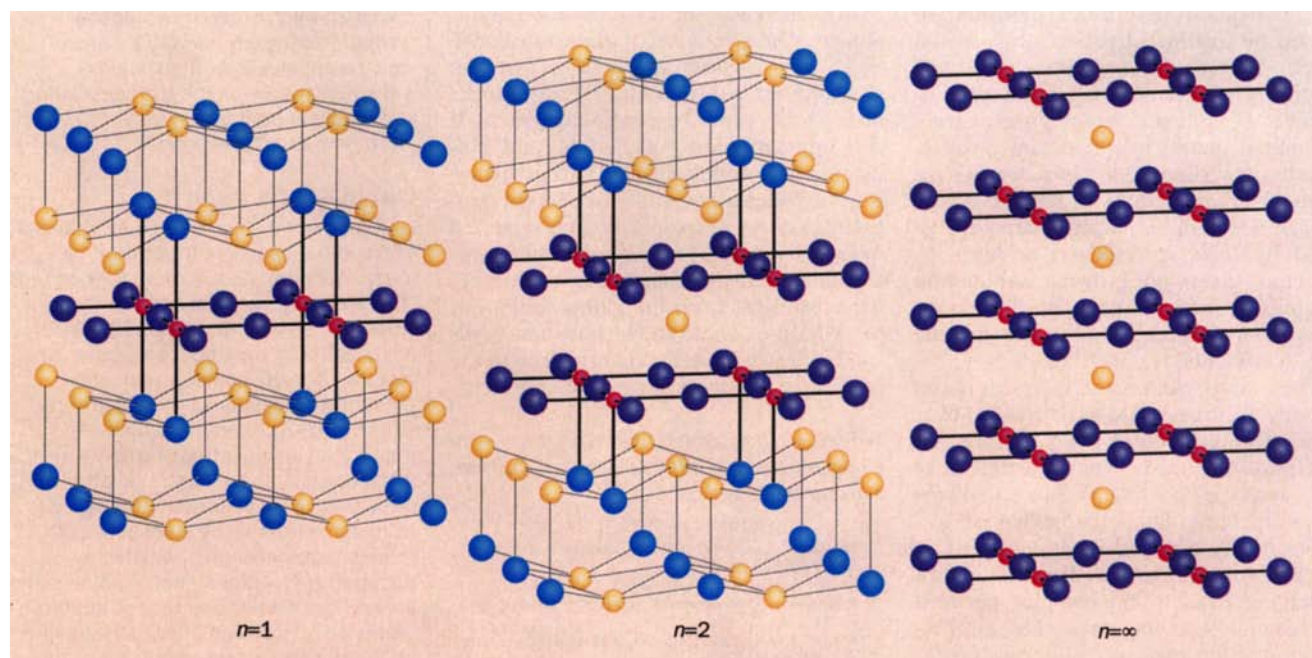
With up to half of the oxygen atoms missing from the $\text{Sr}_2\text{O}_{1+\delta}$ layers, it is clear that, whatever the exact structure of these layers may be, all of the copper atoms in the neighbouring CuO_2 planes cannot have apical oxygen atoms. So homogeneous apical oxygen coordination is not, as has previously been assumed, a requirement for superconductivity in hole-doped copper-oxide compounds.

These compounds may well have been seen before. Adachi *et al.*² observed phases that had essentially the same c -axis repeat distances and the same transition temperatures, present as intergrowths in 'infinite-layer' ($\text{Sr}_{1-x}\text{Ca}_x$) $_{1-y}\text{CuO}_{2+\delta}$ compounds ($x \approx 0.3$, $y \approx 0.1$, $\delta \approx 0.1$) formed at high pressure², but were unable to determine the structures and compositions of the new phases uniquely because of the multi-phase nature of their samples. They speculated correctly, however, that the phases they were seeing were members of a new and rather simple homologous series.

If these strontium-rich phases can exist as intergrowths in infinite-layer compounds, this raises questions about the doping mechanism for such compounds. The limit of the $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ series is SrCuO_2 with the 'infinite layer' structure, as shown in the figure. The infinite-layer structure is particularly appealing because of its simplicity. It contains the only structural elements believed to be essential for high- T_c superconductivity (the CuO_2 layers) with a minimum amount of metal-ion spacer in between. In addition, it is the only structural type in which both electron and hole doping can induce superconductivity^{3–7}.

But whereas the electron doping (T_c up to 43 K) is simply achieved by replacing strontium with a lanthanide element, while maintaining an otherwise perfect crystal structure⁸, the source of hole doping, which induces the highest transition temperatures (T_c up to 110 K), remains a mystery. In addition, hole-doped finite-layer superconductors always have small diamagnetic signals ($\sim 5\%$), suggesting that only a small part of the sample may be superconducting.

Lattice images from high-resolution electron microscopy of hole-doped infinite-layer samples reveal planar defects randomly distributed throughout the crystal. Hiroi and co-workers extensively studied these defects in $(\text{Sr}, \text{Ca})\text{CuO}_2$, and concluded that they have essentially the same crystal structure as regions without defects, but accommodate a large non-stoichiometry of both (Sr, Ca) and oxygen⁹. The planar defect they observed



Structures of the $n = 1$, $n = 2$ and $n = \infty$ members of the $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ series. Strontium, copper and oxygen atoms are shown in yellow, red and blue, respectively (with light blue indicating that the oxygen sites in the $\text{Sr}_2\text{O}_{1+\delta}$ layer are only partially

occupied). Higher-order members of the series can be obtained by inserting more and more $\text{Cu}_2\text{—Sr}$ layers between the $\text{Sr}_2\text{O}_{1+\delta}$ blocks. For $n = \infty$ only CuO_2 and Sr layers are present (figure courtesy of J. Rodriguez).

is not to be confused with a $\text{Sr}_2\text{O}_{1+\delta}$ layer, as it has a different stacking arrangement. According to the authors, the charge balance between metal-ion and oxygen vacancies allows these defect layers to act as a doping mechanism for the adjacent CuO_2 layers. However, an unambiguous connection between these planar defects and superconductivity has yet to be proven.

The discovery of the $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ phases suggests an entirely different explanation for the doping of infinite-layer compounds. As the in-plane lattice parameter changes very little with n , we can almost guarantee that there will be intergrowths of various members of the series with the infinite-layer phase, as observed by Adachi *et al.* Could the small amounts of intergrowth regions be responsible for superconductivity in 'pure' infinite-layer materials? From the present standpoint, we might conclude that either or both of these defects, occurring randomly, could act as a doping mechanism in infinite-layer compounds. Considerable effort will be required to settle this question.

The fact that pressures more familiar to geologists than chemists are needed for bulk synthesis of the new compounds should not discourage the quest for practical applications. Thin-film technology

provides a very effective way of applying 'pressure' on crystal structures, because the film attempts to match the lattice parameter of the substrate. Critical temperatures as high as 150 K have been reported for infinite-layer films¹⁰. Although these extraordinary results have not been reproduced by other laboratories and are controversial, the prospect of high T_c in a binary oxide film is too appealing to be left untried. □

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CELL BIOLOGY

Fast forward to fusion

Dan Cutler

THESE are the best of times for people trying to find out what goes on when biological membranes fuse, a process central to cell function. Scarcely a month goes by without some striking development or other, and one such offering for July can be found elsewhere in this issue.

The transport of proteins between organelles is largely carried out by vesicles, and components of the molecular machinery involved in the docking and fusion of vesicles with their target membranes are being rapidly identified. On page 346 McMahon *et al.*¹ report the preliminary characterization of a new protein, cellubrevin, involved in these events.

Cellubrevin was found by screening a genomic library for homologues of the synaptic vesicle membrane protein synaptobrevin (also known as VAMP). The demonstration that synaptobrevin is degraded by tetanus and botulinum neurotoxins^{2,3} implicated it in the fusion of synaptic vesicles with the presynaptic plasma membrane. This naturally led to speculation that other membrane-fusion events in vesicular traffic would each involve a homologue of synaptobrevin. Hence the excitement over the identification of cellubrevin, which seems to be one

of those homologues.

Like its neuronal counterpart, this new ubiquitously distributed protein can be degraded by the tetanus toxin both *in vitro* and *in vivo* (by transfection of a cell line with a complementary DNA encoding the tetanus toxin light chain). The functional consequences of this proteolysis are potentially fascinating but have yet to be determined. Nonetheless, some insight into the cellular role of cellubrevin comes from the use of antibodies raised against it: they co-localize with antibodies to the transferrin receptor, a membrane protein that constitutively recycles between the plasma membrane and the interior of the cell where it is found in endosomes.

While this particular story has been unfolding, however, two other approaches to vesicular transport have begun to transform the context in which the description of cellubrevin will be seen.

One has involved genetic studies in the yeast *Saccharomyces cerevisiae*, which have led to the identification of proteins related to synaptobrevin as well as to proteins related to syntaxin; syntaxin is found on the presynaptic plasma membrane with which the synaptobrevin-containing membrane fuses. Mutations

in the genes encoding these yeast proteins block specific steps in the secretory pathway, suggesting that both a synaptobrevin-like and a syntaxin-related protein^{4–6} may be involved at each stage of vesicular transport.

The second approach has been biochemical. Rothman and colleagues have demonstrated that a 20S cytoplasmic protein complex, which is required for the fusion of a transport vesicle to a target membrane, can bind to both syntaxin and synaptobrevin⁷. It could therefore act in linking the synaptic vesicle to the target membrane. They have proposed the name SNAREs for these membrane proteins — v-SNAREs (such as synaptobrevin) for those on transport vesicles, and t-SNAREs (such as syntaxin) for those on the target membrane. In this terminology, cellubrevin may be a v-SNARE involved in a fusion event in all cells, just as synaptobrevin is involved in regulated fusion between the plasma membrane and synaptic vesicles in neurons.

One reason why the new paper¹ is so interesting is the co-localization of cellubrevin with the transferrin receptor. This tells us that cellubrevin is present throughout transferrin-containing endosomes, an extremely complex and plastic endomembrane system that may be subdivided into organelles linked by vesicular traffic. So cellubrevin could be involved in fusion between incoming vesicles and the endosome, in transfer between endosomal subcompartments, or in the fusion of recycling vesicles with the plasma membrane. If it is present throughout the endosome, then does it contribute to more than one fusion event, or is location not a good indicator of activity? Fortunately, cellubrevin's toxin-sensitivity should in principle allow the site at which it acts to be determined. Indeed, if many v-SNAREs prove to be sensitive to tetanus and botulinum toxins then, even without a genetic approach, answers to some of the questions of v-SNARE function in mammalian cells may not be far away.

The findings of McMahon *et al.* also support the view that the synaptic vesicle may be just a specialized version of transport vesicles travelling between the endosome and the plasma membrane. If the synaptic vesicle membrane protein synaptophysin (also known as p38) is expressed in cells that lack other synaptic vesicle components, it also co-localizes with the transferrin receptor (see, for example,

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ref. 8). Moreover, antibodies raised against synaptobrevin also recognize vesicles in adipose cells that contain the glucose transporter Glut4 (ref. 9); like synaptic vesicle membrane proteins, Glut4 appears on the plasma membrane when triggered by extracellular factors.

Together, these results imply that there is a close relationship between synaptic vesicles, Glut4 vesicles and the constitutively recycling transferrin receptor-containing vesicles in which cellubrevin appears to be located. If so, then the endosome begins to emerge as an important regulator of cell-surface expression,

able to influence both import and export processes.

Finally, the paper by McMahon and colleagues suggests that every cell — including yeast — could potentially fall victim to tetanus toxin. Fortunately, the restricted distribution of surface receptors has precluded such a dramatic demonstration of the importance of membrane traffic. □

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HUMAN GENETICS

Sexual orientation and the X

Mary-Claire King

LAST week, Dean Hamer and colleagues described a linkage analysis of homosexual brothers which suggests the existence of a gene on the X chromosome that influences male sexual orientation (*Science* **261**, 321–327; 1993).

The first point to be stressed is that this result is preliminary. The evidence is based on a small, highly selected group of homosexual men. The result is purely statistical. The gene is hypothetical and has not been cloned, and the linkage has been observed in only one series of families. Were virtually any other trait involved, the paper would have received little public notice until the results had been independently confirmed. But the trait is homosexuality. At a time when the issue of whether gays and lesbians have the right openly to join the US armed forces is at the forefront of the political agenda, and homophobia is the best fund raiser for the radical right since the demise of Communism, it is no surprise that the possibility of a 'gay gene' has received enormous attention.

What exactly are the data? They come from genetic analysis of 40 pairs of homosexual brothers who had identified themselves as gay, had extreme scores on Kinsey scales of sexuality, and had volunteered themselves (and in 14 families, their mothers as well) for the project. They were not, and were not intended to be, representative of all gay men. The sample was intentionally selected to give the best chance of detecting a genetic influence on sexual orientation.

Genetic markers on the X chromosome were typed for each pair of brothers and (when available) their mothers. For any X-linked genetic marker, the chance that two brothers share the same allele by descent from their mother is 0.5. If pairs of brothers inherit the same maternal X-linked alleles more frequently than expected by chance, it suggests that the

brothers share a trait due to a gene on the shared portion of their X. These 40 pairs of brothers inherited the same maternal alleles only as often as expected for most of the length of the X chromosome. But for markers at Xq28, 33 of the 40 pairs inherited the same alleles from their mothers; seven pairs of brothers were discordant for their maternal alleles at those markers. The difference between the ratio of the 33 concordant to 7 discordant pairs observed, and the 20 concordant to 20 discordant pairs expected, is significant at a probability of better than 0.001. In the published paper, this result is expressed in the terminology of genetic mapping as a lod score of 4.0 in favour of linkage of homosexuality to a locus at Xq28 in these families.

Whether the statistical significance of this linkage represents a gene for sexual orientation will be clear only after more families have been evaluated. Several times in the recent history of human gene mapping, linkage results at this level of significance have turned out to be artefacts of poor experimental design or of coincidence.

To the credit of Hamer and his colleagues, however, in their study they have addressed most of the problems of evaluating linkage to complex traits. Only families with well-defined pairs of homosexual brothers were included. Also, because the frequency among heterosexuals of (unexpressed) alleles for homosexual orientation is completely unknown, and incomplete penetrance of the homosexual phenotype would confound the analysis, heterosexual siblings were not included in linkage. Furthermore, in order to minimize heterogeneity among causes of homosexuality, families with only one homosexual man, more than one lesbian relative, or any father-son pairs of homosexual relatives were excluded. Finally, many markers on the X chromo-

some were genotyped, with the expectation that recombination of maternal X chromosomes would yield at most only a small region of the X held in common by the homosexual brothers. The other X chromosome markers served as an internal control. So the sample of families and markers used for linkage analysis was designed to maximize the chance of detecting any X-linked genetic influence, and successfully so.

The clue that a gene on the X chromosome might influence sexual orientation in males came from family histories reported by 76 homosexual men, which the authors obtained before undertaking the linkage analysis of brothers. These men reported homosexuality to be significantly more common among their maternal uncles compared with their paternal uncles, and among their male cousins through maternal aunts compared with their other male cousins. I find these family history data precarious. It could be that maternal male relatives were more willing to reveal their homosexuality than were paternal male relatives. Independent assessment of sexual orientation among the 655 nominally heterosexual relatives was extremely limited: only 30 answered the Kinsey questionnaires. Under-reporting of homosexuality, particularly among paternal male relatives, therefore cannot be excluded. However doubts about reporting bias do not apply to the linkage analysis, because only brothers identified as homosexual by self-report and Kinsey scores participated in that part of the study.

It is impossible to use either the family history or the linkage data to estimate the magnitude of a genetic influence on homosexuality in the general population. The family history survey was not designed to separate social and genetic influences on homosexuality. Molecular analysis was undertaken only for the 40 pairs of homosexual brothers, and because the brothers were selected in such an (intentionally) biased way it is impossible to generalize from them. The X-linked locus could tell us nothing about male homosexuality (if the result is a statistical fluke not replicable in other families); it could have a very small influence (pertaining only to men from families like those in the linkage study); or it could be important (if homosexual men generally are ultimately shown to carry the same alleles as the brothers in the selected families).

Among the questions that cannot yet be answered are the following. What fraction of homosexual men (not necessarily with homosexual brothers) carry an X-linked allele influencing sexual orientation? How frequent is this allele in the heterosexual population (in other words, what proportion of men carrying the allele are not homosexual)? How many different alleles are there at this still-hypothetical locus, and what proportion of men with each

allele are homosexual? And how many other genes influence sexual orientation for men or women? Both replication of the present results, and other experimental designs, will be required to answer these questions.

The consequences of ultimately identifying a gene or genes influencing sexual orientation would be considerable. On the one hand, identification of genetic influences on homosexuality would support the hypothesis that sexual orientation is a continuous trait with many biological and social influences, and normally distributed variation between complete homosexuality and complete heterosexuality. (Sexual orientation may be like handedness. We may be right-handed or left-handed. The hand we favour may be genetically influenced, as well as open to alteration by environmental pressures. We may also vary in our capacity for ambidexterity or single-handedness. This capacity for ambidexterity may also be genetically influenced, possibly by different genes than those that affect hand preference.) In this context, evidence that sexual orientation is genetically influenced would undermine the argument that homosexuality is a moral choice. Rather, it would be established as a biological reality, just as heterosexuality is.

On the other hand, it would be naive to ignore the possibility, however remote, of prenatal identification and abortion of male fetuses carrying alleles influencing homosexuality. Sexuality — that is, being female — is now the world's most common reason for elective abortion based on a genetic trait. I would like to believe that, through education, we could prevent the emergence of a comparable level of intolerance against homosexual males. But, like any fundamental advance in human understanding, modern genetics is vulnerable to misappropriation.

The potential for abuse might arise in part from mistakenly equating genetic analysis with genetic disease — undertaking genetic analysis of sexual orientation might be taken to mean that geneticists consider homosexuality a 'disease', and that if genes for sexual orientation exist, their 'abnormal mutations' could be 'fixed by gene therapy'. This would be a misinterpretation; only a small fraction of the genetic variation in the human genome is associated with disease. The revelation of the extraordinary genetic diversity of normal human characteristics is one of the most fascinating results of modern human genetics. To identify variation and characterize genetic influences on it is to recognize the significance and value of that variation, not to condemn it. □

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Why introns-in-pieces?

Andrew J. Roger and W. Ford Doolittle

ON page 358 of this issue¹, Ferat and Michel provide evidence for the existence of self-splicing group II introns deep within the bacteria. Group II introns (which differ from other introns in structure and in the mechanism of splicing) are found, it was thought, only in chloroplast and mitochondrial genomes. Not so, it turns

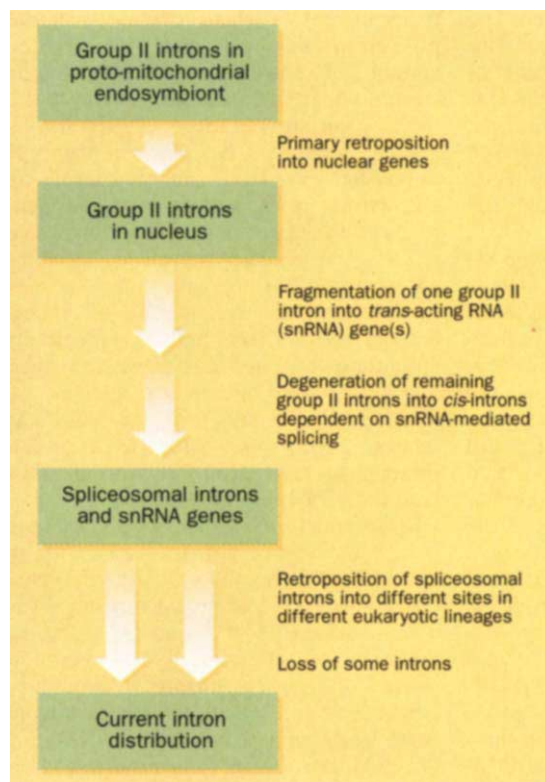
evolution, coupled with those of Darnell and one of us on their role in the primordial assembly of genes³, became widely known as the 'introns early' theory — genes were split from the outset, eukaryotes have retained many of their original complement of intervening sequences, and prokaryotes have lost them through genome 'streamlining'.

Opposing ('introns late') theories held that introns were selfish elements which spread into eukaryotic genes after the origin of the nucleus. In 1991, Cavalier-Smith formulated an appealingly testable introns-late scheme, which holds that nuclear spliceosomal introns descend from group II self-splicing introns initially residing in the genome of the α -purple bacterial endosymbiont which gave rise to the mitochondrion⁴. Such organellar introns, he proposed, invaded nuclear protein-coding genes by retroposition. Subsequent fragmentation of one of these selfish elements produced genes for small RNA species — the ancestors of small nuclear (sn) RNAs — which could splice all of the group II introns present in the nuclear genome *in trans*, making autocatalysis redundant.

Cavalier-Smith's hypothesis rests on four propositions. First, that spliceosomal introns are really just 'introns-in-pieces' fragmented from a self-splicing group II intron ancestor; second, that group II introns can be retrotransposable elements; third, that group II introns were present in the bacterial ancestor of the mitochondrion; and, fourth, that both kinds of introns are absent in the eukaryotic lineages that diverged earliest,

which never had mitochondria. Recent discoveries have added credibility to the first three of these contentions.

The claim that spliceosomal introns are fragmented group II introns is now supported not only by similarities in splice-site sequences and lariat production, but by detailed resemblances between intermolecular base-pairing interactions of snRNAs with each other and with spliceosomal intron/exon sequences and intramolecular base-pairing in group II introns^{5,6}. Further evidence comes from the observation that group II introns can



Possible explanation of the origin and subsequent spread of spliceosomal introns in eukaryotic genomes. Group II introns first entered eukaryotes in the genome of the purple bacterium which became the mitochondrion. The retroposition of these introns into the nucleus and the subsequent fragmentation of one of them into separate snRNAs and introns produced spliceosomal introns and the splicing machinery. Slow spreading of spliceosomal introns has since occurred by reverse-splicing, reverse transcription and recombination, generating newly split genes. The current intron distribution reflects both this kind of intron spread as well as some intron loss.

out, for Ferat and Michel have now identified them in the putative bacterial fore-runners of these organelles. This announcement will cause a stir, for it has exciting implications for our understanding of intron evolution.

In 1978, when his "Why genes-in-pieces?" article was published in these pages², Walter Gilbert tabled the first argument in a continuing debate on the origins of introns (more precisely of the 'spliceosomal' introns which disrupt nuclear protein-coding genes). Gilbert's ideas about how such introns could 'speed'

be fragmented into pieces *in vitro* without losing spliceability⁷, and that they have been fragmented in the evolution of several independent organelle lineages⁸.

The second claim, that group II introns are mobile, was first prompted by the finding that many bear reverse transcriptase-like open reading frames (ORFs)⁹, and the subsequent demonstration of reverse splicing *in vitro*¹⁰. Together these suggested a mechanism of spreading into new genes by reverse splicing into heterologous RNAs, reverse transcription and recombination of the complementary DNA with the intronless copy. The case for group II mobility now seems to have been sewn up by the report of actual transposition of these introns into new sites in the yeast mitochondrial *cox1* gene (probably by an RNA-based mechanism) (M. W. Mueller, personal communication) and the finding that the protein encoded by one of the intron ORFs is an intron- and exon-specific reverse transcriptase¹¹.

Evidence for the third claim has been longer coming. Cavalier-Smith proposed his theory under the influence of a preliminary report that group II introns had been found in bacteria¹² (which was not then confirmed). So people could still hold — until they read this issue — that group II introns arose afresh in organellar genomes too recently to have sired the spliceosomal introns of all eukaryotic nuclear genes. Ferat and Michel's report of the discovery of group II introns in eubacteria¹ now makes it again reasonable that introns first entered eukaryotes in the ancestors of organelles.

Ferat and Michel designed PCR primers for the conserved domain of the characteristic reverse transcriptase-like ORF and the conserved domain V element of the intron secondary structure. By screening a variety of cyanobacterial and purple bacterial DNAs, they were able to clone and characterize two intron fragments from the cyanobacterium *Calothrix* and one from the γ -purple bacterium *Azotobacter vinelandii*. Sequencing of a cyanobacterial intron showed all six characteristic group II intron secondary structural elements, as well as the reverse transcriptase-like ORF. If we accept that the common ancestor of both of these bacterial lineages must then have had group II introns, such introns are indeed older than organellar genomes, and might well have first entered eukaryotic cells through the endosymbiotic eubacterial ancestor of mitochondria.

This leaves the fourth claim — that eukaryotes that never had mitochondria don't now have spliceosomal introns. To date, about a dozen genes have been shown to be intronless in the archezoan *Giardia*, which is thought to lack mitochondria primitively. However, a comparably large sample of genes from

Saccharomyces might also reveal no introns — so we do need more data. The finding of a single group II or spliceosomal intron in any *bona fide* archezoan, or the demonstration that these contain spliceosomal snRNA genes, would be sufficient to render the mitochondrial origin theory unnecessary.

Cavalier-Smith's scheme nicely rationalizes the origin of spliceosomal introns, but we suggest that it does not explain their spread within nuclear genomes. The fact that homologous genes in evolutionarily distant eukaryotes have many introns of which few are in shared positions points not only, as is often assumed, to intron loss, but also to the continuous insertion of new introns since their first appearance in eukaryotic nuclear genomes. No nuclear group II intron has ever been found, whereas all eukaryotes with mitochondria appear to have the machinery for spliceosomal splicing. It seems, therefore, simplest to suppose that this machinery is also responsible for the spread of spliceosomal introns (see figure). One likely spreading mechanism, suggested long ago by Sharp¹³, is reverse splicing (at some low frequency) into new RNAs bearing proto-splice sites. In a similar manner to their group II cousins, they could then be integrated into the genome.

To support such a theory we need both *in vitro* evidence for the feasibility of reverse spliceosomal splicing, and convincing examples of recent intron gain *in vivo*, where the mechanism might be deduced by identifying the genomic source of the new intron. It should be possible to find examples where introns have been gained recently in evolution (cases where several outgroups in a clade lack an intron which a single member exhibits). The relationship between the new intron and its progenitor should, we calculate, certainly be detectable by hybridization for insertions occurring since the divergence of the apes from humans¹⁴, and by sequence for more ancient events.

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Mental strife

THE technological predictions of the sixties read very strangely now.

Computer speed and memory were already doubling every year. Soon, said the pundits, computers would overtake the human brain itself, whose huge storage space is offset by its dismally slow processing speed. The near future would be dominated by wonderful, flexible, linguistically skilled, intelligent computers.

What went wrong? The speed and power of computers increased, and is still increasing, just as rapidly as those early experts predicted. But computers are still as wooden and stupid as they ever were. They are just wooden and stupid in 128 pointless fonts and 16 million indistinguishable colours.

Many designers are now hoping to create an intelligent computer by imitating the brain's supposed neural-net architecture. But Daedalus thinks the secret lies in the division of the human mind into superego, ego and id. He recalls those cunning 'artificial life' programs which compete against each other in a computer. They evolve amazingly rapidly into the most subtle and non-intuitive forms. The development of human intelligence may similarly occur by internal competition between the three mental entities. So DREADCO's new learning system mirrors the human mind. It has three competing sub-programs. Its 'ego' interfaces with the real world, and handles most calculation; the 'id' has many random overrides and interrupts; the 'superego' keeps memory locations and processing-time tightly allocated. Each has its own niche in programming space. Each can be challenged by the other two, but cannot be driven extinct.

The goal of the whole system is simply to learn the tasks chosen by its human master. The initial version was quite as babyish and exasperating as any modern dinosaur of programming. But gradually, by a mixture of competition and cooperation, the three sub-programs are learning by experience. Sometimes they steal or sabotage each other's code, sometimes they delegate tricky tasks to each other — rather like the neurotic but creative confusion in the human mind itself. Soon the joint system should be educated enough to be useful. When it is as tractable as its rival conventional programs, DREADCO will bring it to market. It will go on evolving in the hands of the buyer, depending on what he wants from it. It should ultimately become his general intellectual servant: a true 'personal digital assistant', to borrow a phrase from the latest generation of programming dinosaurs. David Jones

Viral burden and HIV disease

SIR — The central paradox of human immunodeficiency virus (HIV-1) infection has been the apparent paucity of infected cells¹ relative to the functional defects² and progressive CD4⁺ cell loss which occurs during the asymptomatic phase³. The authors of recent articles published in *Nature* and *Science* claim to have resolved this paradox by finding "massive" infection of lymph node cells^{4,5}, and very high levels of virion RNA in plasma⁶. In addition, it has been suggested in two editorials that these findings bolster the credibility of a simple cytopathic model for AIDS pathogenesis^{7,8}. But, in our view, these new findings do not justify this conclusion for several reasons.

First, although the frequency of infected cells may be 5- to 10-fold higher in lymph nodes than in peripheral blood⁴, most of this infection appears to be latent⁵. In a cytopathic model, the rate of CD4⁺ cell loss would be determined by the number of actively infected cells, and even a frequency of one per 100 lymph node cells (based on 1/10 infected and 90 per cent latent), is still minuscule compared to the regenerative capacity of the immune system. In addition, presenting such results as the proportion of infected cells can be misleading. For example, the one late-stage patient, as described by Pantaleo *et al.*⁴, had a 10-fold higher ratio of infected to uninfected peripheral blood cells but also had a 10-fold lower number of CD4⁺ cells. Thus the absolute number of infected CD4⁺ cells per mm³ was similar to that found in asymptomatic subjects.

Second, the high level of plasma virus observed by Piatak *et al.*⁶ was about 99.9 per cent non-culturable, suggesting that it was either neutralized or defective. Therefore, rather than supporting a cytopathic model, this observation actually may help explain the relatively slow dissemination of the infected cell burden and thus the relative ineffectiveness of therapy with nucleoside analogues which target this process.

Third, we question the longitudinal conclusions some of these investigators have drawn from cross-sectional data. The results presented are equally consistent with the conclusion that higher viraemia is a consequence of, rather than the proximate cause of, defective immune responses.

Finally, the cytopathic model predicts that HIV-1 variants with increased cytopathicity would produce more rapid CD4⁺ cell loss. Another study, recently published in *Science*, reported exactly the opposite result when Hu-PBL SCID mice were infected with phenotypically divergent HIV-1 isolates⁹. Taken at face value,

this finding is more consistent with the conclusion that such variants occur as a late consequence of immune system failure.

Instead of showing us "where all the cytopathic HIV has been hiding", recent results⁴⁻⁶ only strengthen the conclusion that most of the progression to AIDS occurs in the presence of a very low level, albeit chronically active, HIV-1 infection which disrupts and gradually destroys the immune system by mechanisms which cannot be fully accounted for by a direct virus-mediated cytopathology. Therefore, we continue to stress the importance of investigating alternative mechanisms which can account for HIV-1 disease progression in the light of this central paradox¹⁰.

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FAUCI ET AL. REPLY — In our studies of HIV infection in lymphoid tissues^{4,5} we found that: (1) even at relatively early stages of infection, large intracellular reservoirs (CD4⁺ lymphocytes and macrophages) have been established as one mechanism by which HIV can elude host defences; (2) extracellular reservoirs (follicular dendritic cells) have also been established from which infection can potentially spread by transmission of virus to migrating CD4⁺ cells; and (3) there is always some viral gene expression from the clinically latent to clinically apparent stages of infection, with increasing gene expression and viral replication at later stages. Sheppard *et al.* express concern that these and other studies⁶ have been incorrectly interpreted^{7,8} as support for a simple cytopathic model for AIDS pathogenesis; however we make no claim to have identified precise mechanisms whereby CD4⁺ T cells are killed. What we can say is that with such a large number of CD4⁺ lymphocytes infected, the ongoing activation of replication in even a small fraction of infected cells could gradually eliminate a substantial segment of the population, either as a direct consequence of viral replication or by indirect mechanisms (for example by an immune attack on infected cells with active viral gene expression). In addition, the persistence of a replicating virus could trigger a complex series of immunopathogenic events even involving uninfected cells, which could account for the progressive deterioration of the immune system.

With regard to the specific points which Sheppard *et al.* make regarding our papers, they have misinterpreted our

point⁴ made with the polymerase chain reaction (PCR) data in late- versus early-stage patients. We performed PCR on unfractionated peripheral blood and lymph node mononuclear cells. The late-stage patient (patient 10) had a 10-fold higher frequency of infected cells in unfractionated mononuclear cells than did an earlier-stage patient (patient 6). Patient 10 had 42 CD4⁺ T cells per mm³ whereas patient 6 had 355 CD4⁺ T cells. Sheppard *et al.* state that the later-stage patient "also had a 10-fold lower number of CD4⁺ cells. Thus the absolute number of infected CD4⁺ cells per mm³ was similar to that found in asymptomatic subjects". This is incorrect. Because patient 10 had only 42 CD4⁺ T cells per mm³, the 10-fold higher signal in a given number (100,000) of unfractionated mononuclear cells compared to the signal in a comparable number of unfractionated mononuclear cells from patient 6 who had 355 CD4⁺ T cells indicates the opposite of what Sheppard *et al.* contend. Because approximately 10-fold less CD4⁺ T cells in the fraction from the late-stage patient gave a 10-fold higher PCR signal than that demonstrated in the mononuclear cell fraction from the early-stage patient, this means that a far greater number of CD4⁺ T cells plus probably other CD4⁺ cells had to be infected in the late-stage patient.

With regard to the comments of Sheppard *et al.* on work by others^{6,9}, we can only give our own brief interpretation of how this relates to our studies. Our interpretation of ref. 6 underscores the concept that there is far more virus in the body than was formerly appreciated. That paper does not address mechanisms of cell killing discussed above. The main message of ref. 9 might be that caution should be exercised in extrapolating *in vitro* results to the *in vivo* situation with regard to the degree of cytopathicity of HIV strains; furthermore, it might also caution us in the extrapolation of results from the Hu-PBL SCID mice to humans. Here again, these studies do not address the complex mechanisms of HIV-related destruction of the immune system.

The data in one of our papers⁴ indicate that there is a progressive deterioration of the architecture and microenvironment of the lymphoid tissue, and in particular a gradual dissolution of the follicular dendritic cell network of the lymph-node germinal centre. Because follicular dendritic cells and bone-marrow-derived dendritic cells and macrophages are important antigen-presenting cells to B and T cells^{11,12}, the functional integrity of these cells is critical for the generation of an effective immune response. Our data indicate that follicular dendritic cells are probably killed by mechanisms that are not limited to direct virus-mediated cytopathicity. And so here too this major lesion in the immune system is not neces-

sarily directly caused by virus-mediated killing, but probably involves mechanisms related to the presence of a high viral burden and persistently replicating virus.

We agree with Sheppard *et al.* that the mechanisms of destruction of the immune system are complex and are certainly not yet fully delineated. We do feel, however, that the virus has a central role in the pathogenesis of HIV disease either directly or indirectly by triggering a series of immunopathogenic events which contribute to the progressive immunosuppression. Our demonstration of high viral burden and active virus replication throughout the course of HIV disease strongly supports this hypothesis.

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Large herbivores in rangelands

SIR — The biomass of domestic ungulates in South American rangelands is higher, at a regional scale, than the biomass of wild herbivores in a set of natural ecosystems worldwide, and it has been argued that the same is true of Africa¹. Oosterheld *et al.* concluded¹ that animal husbandry practices they described as “elementary” raise the carrying capacity of rangelands for large herbivores. Using statistical analyses on a larger data set, we show here that large herbivore biomasses in comparable pastoral and natural sites of the African savannas are closely similar, implying that the husbandry practised in these pastoral areas has no effect on carrying capacity.

The African savanna biome, with its relatively undisturbed and varied large-herbivore communities, vegetation types and soils, contains one of the largest available sets of data on the abundance of large herbivores in rangelands: it is therefore of particular interest in this

context. Previous studies have established that a major factor influencing the biomass (carrying capacity) of rangelands for large herbivores (via primary production) is rainfall^{2–4} and have implied that soil nutrient availability^{4,5} is another major factor. Here we extend

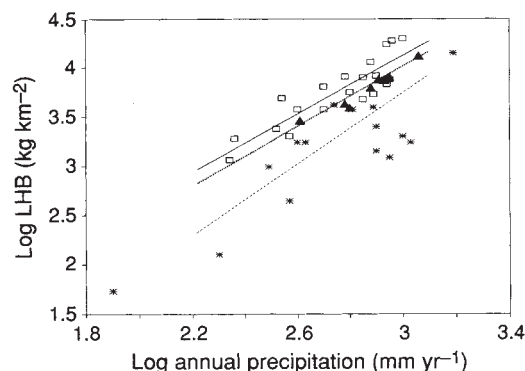


FIG. 1 Relationships between rainfall (annual precipitation, AP, mm yr⁻¹) and large herbivore biomass (LHB, kg km⁻²) for the three soil nutrient availability (SNA) groups: high (□), medium (▲) and low (*). Logarithms are to the base 10. $\log LHB = 1.45 \log AP - 0.236$ ($r^2 = 0.78$, $P < 0.001$, $n = 20$) for high SNA; $\log LHB = 1.50 \log AP - 0.509$ ($r^2 = 0.95$, $P < 0.001$, $n = 8$) for medium SNA; and $\log LHB = 1.55 \log AP - 1.12$ ($r^2 = 0.74$, $P < 0.001$, $n = 16$) for low SNA. Although the slopes are not significantly different (covariance analysis in this and subsequent comparisons, $F = 0.06$, $P > 0.05$, d.f. = 2), the y-intercepts are ($F = 35.4$, $P < 0.001$, d.f. = 2).

these analyses, which included 35 African sites, in two ways: by controlling for the effect of soil nutrients as well as rainfall on herbivore biomasses; and by testing these hypotheses statistically on a larger data set (65 sites).

Our data include the biomasses of domestic and wild large herbivores, rainfall and geomorphological types for sites in Africa south of the Sahara^{2,4,5} (other references available from the authors). Each site was classified for soil nutrient availability (SNA) into one of Bell's broad classes⁵ (high, medium and low); and into pastoral or natural systems. The former are characterized by basic livestock husbandry techniques such as protection from predators, selective breeding and some veterinary care. Some supplementation of water is practised, but food supplementation, irrigation and fertilization of the swards are not. Some of these systems include a significant wildlife component in their total biomass.

We evaluated the effect of soil nutrients on the total biomasses of large herbivores by calculating the relationship between large herbivore biomass and rainfall for the three SNA classes separately. Biomass was, as expected,

positively correlated with rainfall for all three classes (Fig. 1), and the intercept of the high SNA class is significantly higher than medium SNA, which is higher than for low SNA. This result supports the suggestion^{4,5} that soil quality, through nutrient availability, is a key determinant of large herbivore biomass in these ecosystems: for a given amount of rainfall, biomass is about three times greater on high than on low SNA.

In the previous studies^{2,6} it was noted that the total biomasses in pastoral systems were on average higher than in natural systems. But when both soil nutrient status and rainfall are considered on sites where pastoral management is as defined above (basic), this pattern disappears. Within the high SNA class, large herbivore biomass and rainfall are correlated for both natural and pastoral sites (Fig. 2); however the slopes and intercepts are closely similar. The same result is obtained for medium SNA and for low (data not shown).

This study provides, for the first time, statistical evidence for the suggestion^{4,5} that soil nutrient level affects the biomass of communities of large ungulates.

The model is a useful predictive tool for rangeland managers working in this kind of ecosystem.

The analysis also shows that comparisons of livestock and wild herbivore biomasses which do not include the effect of soil quality as well as rainfall are invalid, and that the African pastoral systems studied here do not have higher carrying capacities than natural grazing systems. Pastoral management clearly can affect the carrying capacity of Afri-

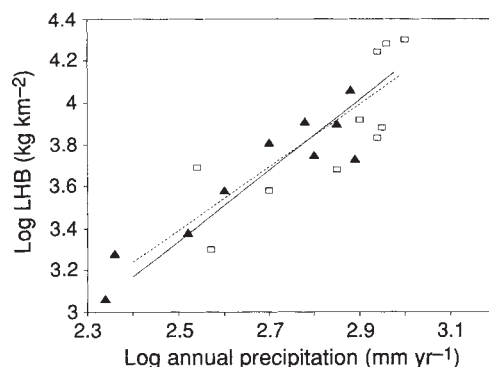


FIG. 2 Relationships between rainfall (AP) and LHB for pastoral (□) and natural sites (▲) with high SNA. The regressions are $\log LHB = 1.56 \log AP - 0.56$ ($r^2 = 0.65$, $P < 0.01$, $n = 10$) for natural; and $\log LHB = 1.41 \log AP - 0.115$ ($r^2 = 0.86$, $P < 0.001$, $n = 10$) for pastoral. The differences are not significant for slopes or intercepts ($F = 0.13$, 0.046 , both $P > 0.05$, d.f. = 1).

can, as well as other rangelands^{1,6}. Further work is required to identify the nature and limits of these effects.

Wildlife is increasingly viewed as a natural resource which merits sustainable use rather than extermination in pastoral grazing systems⁷. For this to succeed, a fuller understanding is required of the natural and human factors regulating the densities, and particularly the productivities, of wild and domestic large herbivores in these ecosystems.

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a regional scale.

The method used by Fritz and Duncan to assess soil fertility is too coarse and subjective to answer quantitative questions about the importance of soil fertility and precipitation on carrying capacity. This classification basically states that volcanic soils are fertile, those derived from 'basement' rock are infertile, and the others are in between. Quantitative assessments of soil fertility comparable to those of precipitation are feasible and should be used to study this important issue.

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Remembering landmarks

SIR — Because the position of an almost infinite number of objects specifies the same point in space, and memory capabilities of animals are probably limited, one expects memory of only a few landmarks to specify points in space for animals. Much current research focuses on the characteristics of such landmarks, and what form the memory takes. Biegler and Morris¹ report an interesting and carefully designed experiment which they claim reveals a fundamental rule used by animals when deciding which landmarks to remember ("if it moves, don't use it"), and that by applying this, animals extract spatial invariance from their environment. Although the hypothesis is plausible, and their results are suggestive, additional controls are needed before this conclusion should be drawn.

For half the rats in Biegler and Morris's experiments, the goal was specified by two distinct landmarks, the locations of which were fixed relative to a square arena. For the other rats the goal was specified by the same two landmarks, in the same geometrical array, but the whole array was randomly positioned in the arena before each trial. For both groups ('fixed' and 'varied', respectively) the arena was kept stationary within a square room. When the animals had been trained to find the goal (a buried food dispenser) they were given tests with either the landmark array, or the food dispenser, removed from the arena. When the landmarks were re-

moved, both groups searched randomly in the arena, suggesting that cues external to the arena were not guiding animals to the goal. When the food dispenser was removed, there was a marked difference between the two groups. The 'fixed' group searched mostly at the goal, whereas the 'varied' group searched mostly around the nearest landmark to the goal. Biegler and Morris concluded that landmark stability is a prerequisite for spatial learning and memory.

However the animals began trials from the midpoints of the sides of the square arena. Thus, subjects in 'fixed' experienced four "starting viewpoints" of the landmark array. By contrast, subjects in 'varied' experienced up to 196 (there were 49 possible locations of the array in the arena). Consequently, 'varied' subjects were frequently experiencing novel starting viewpoints of the landmark array, and even from starting viewpoints that they had seen before, they would have had much less practice than 'fixed' subjects (up to 1/49).

The ability to reach a goal from a novel viewpoint is thought to require special processing abilities^{2,3}. It should not be assumed to be a trivial task, or unlikely to improve with practice. There are thus two explanations for Biegler and Morris's result — theirs, and that 'varied' animals were simply less practised from each starting viewpoint. There are several ways to distinguish between these interpretations. One would be to see if the tendency of 'varied' rats to use the rule "move towards the triangular landmark and search around it" declines with practice. Because landmark stability and starting viewpoint can be manipulated independently, a better way would be to make them independent factors; this would allow their relative effects to be determined.

There are two other reasons for interest in Biegler and Morris's conclusions. First, experiments on insects^{4,5}, birds⁶ and rodents^{7,8} show, contrary to their conclusions, that animals do learn and remember the precise location of a goal using an unstable landmark array. Second, many ecologically important points for animals remain fixed relative to unstable landmark arrays (mouths, nectar and cheese in rat traps spring to mind), and one might expect animals to have evolved the cognitive ability to remember the precise location of such points. Extracting spatial

MCNAUGHTON *ET AL.* REPLY — Fritz and Duncan conclude that African pastoral and natural systems have similar carrying capacities for large herbivores. According to the data in the sources they cite, wild herbivore-soil-rainfall data are on a local basis in game reserves, while livestock-soil-rainfall data are national averages. This will tend to inflate the density of wild ungulates and to deflate the abundance of livestock substantially. This is because areas chosen for game reserves naturally tend to have abundant wildlife, and much of the total land areas of most African countries will not support livestock owing to tsetse fly, lack of reliable water, inadequate amounts or quality of forage, vegetation types which are not conducive to livestock raising, and so on.

In addition, the assignment of a single value for rainfall and soil fertility to an entire nation is highly suspect, particularly given the regional climatic and edaphic heterogeneity of Africa. Vast areas within an African nation may be covered with miombo or desert, where livestock densities approach zero, while livestock herds are confined to only a small portion of the nation. We conclude that the data presented by Fritz and Duncan are biased and cannot be used to test the effect of animal husbandry on carrying capacity at

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invariance may well be important for animals, but it has not yet been shown to have a preeminent role in determining which landmarks are remembered.

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MORRIS REPLIES — Bennett offers two criticisms of our proposed principle of 'landmark stability'. First, we have not equated the starting viewpoints between our groups; and second, other data indicate that animals can search appropriately with unstable landmark arrays. Although both criticisms are relevant, we doubt the significance of the first, and the second points to an important difference between our own and other studies.

Throughout training in our experiment, all rats frequently ran around the side-walls before venturing into the central region from a point different from that at which they had first been placed. Accordingly, we doubt that the four nominal starting positions have any special status to the rats of either group. We would also draw attention to a study in the water maze (experiment 2 of ref. 1), where the starting-point issue was systematically investigated and shown to be without influence. Further, Bennett's calculations are incorrect because the relative positions of two landmarks, L+ and L- (as defined in our paper), were also changed randomly in group 'varied' rather than the whole array being translated randomly as he assumes.

A key difference between our own and other studies which have looked at unstable landmark arrays is that, with one exception², ours is the only study to specify the position of hidden food in relation to a single unstable L+ landmark. This is potentially different from the situation where two or more L+ landmarks are present because, in this case, animals can form a stable self-contained geometric 'fragment'³ which could represent the position of the hidden food irrespective of lateral translation. Thus, if there is local landmark stability, global stability may not be required. This resolves the apparent contradiction between Bennett's and our own results.

The results of several other experiments support our interpretation⁴⁻⁶, although clearly it would be valuable to compare, with appropriate controls, the single-landmark and two-landmark case. Such an experiment is presently underway. We would not regard finding an accurate search for accessible hidden food (F+) in relation to a moving two-landmark array as an exception to our principle of landmark stability for precisely the reasons given above.

Much work is required to put the notion

that spatial learning involves the extraction of geometric invariance on a firmer footing, or to disprove it, but we believe that our experiment has revealed an unexpected dissociation of fundamental relevance to the concept.

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Structure near the mantle's base

SIR — I am pleased to see that interest in resolving the fine structure of the D" region of the mantle using short-period P waves has been revived by Vidale and Benz¹. However, in an accompanying News and Views article², Wyssession indicates that the presence of a layer of seismically fast material 130 km thick and 300 km wide at the base of the mantle inferred by Vidale and Benz¹ is an unexpected feature. Later, in reference to the high velocity layer, he comments: "The results are all the more intriguing because they do not occur with previous studies".

These statements are both incorrect and misleading. Evidence for the existence of such a layer in D" has been published several times during the past 30 years³⁻⁷, starting with the work of Carder³. These earlier studies suggest that such a high-velocity layer is widespread in the sense that its presence has been inferred in several widely separated regions of the deep mantle⁴⁻⁷. Clarification with a large dataset of high quality¹ is therefore a valuable contribution to our understanding of mantle dynamics. I emphasize that the high-velocity structure implied by the data of Vidale and Benz¹ for paths from China to North America is no different from that implied by our data⁷ for paths from Asia to Australia.

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Alzheimer's response

SIR — The Scientific Correspondence¹ from Kruck contains several inaccurate statements about our Letter². First, the purpose of our report was the analysis of neuritic plaque cores in brains from people with Alzheimer's disease. The elemental composition of neurofibrillary tangles remains to be investigated.

Second, Kruck is mistaken in the view that we have attempted to make any correlation between the presence of neuritic plaques and the severity of dementia. On the contrary, we state that to arrive at a neuropathological diagnosis of Alzheimer's disease "a minimum density of neurofibrillary tangles and neuritic plaques" has to be detected. The presence of plaques is a prerequisite for making a diagnosis.

Third, Kruck has failed to read our paper properly, for he refers to "Their specimen, stated to be from the brain of a patient with Alzheimer's", which ignores our clearly stated and tabulated results of the analysis of neuritic plaques in stained tissue from five, and unstained brain from four, Alzheimer's disease cases.

Fourth, Kruck alludes to our use of particle-induced X-ray emission (PIXE) but fails to recognize the significance of our use of this technique in combination with scanning transmission ion microscopy, which permits the recognition of a unique 'fingerprint' to identify, localize and elementally analyse neuritic plaque cores in unfixed, unstained brain tissue, for the first time. Kruck's comment concerning the absence of information in the report about the use of positive cellular matrix-bound control samples shows a lack of understanding about the physical mechanism underlying the PIXE technique. PIXE uses the removal of inner-core electrons and therefore the analysis is independent of the type of organic matrix being investigated.

Finally, we reiterate our opinion that until other researchers investigating the purported role of aluminium in the pathogenesis of Alzheimer's disease by the use of elemental microanalytical techniques address the issue of artefactual introduction of aluminium into brain tissue by the use of tissue fixatives and stains, controversy will persist over whether this element colocalizes in neuritic plaque cores, neurofibrillary tangles, or within neuronal compartments or organelles.

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Progressive knowledge

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The Advancement of Science. By Philip Kitcher. Oxford University Press: 1993. Pp. 493. \$39.95, £30.

THE unreformed 'legend' of a noble, rational, progressive, truth-discovering science is now celebrated only on high holy days in the most secluded temples of learning. Philip Kitcher here proposes a revised version that can publicly resist the debunking of philosophers and sociologists. The mission will earn cheers in some circles, but scorn in others. It is a serious, learned, thoroughly argued work, which certainly deserves attention by both parties. It claims to bring good news, but will it succeed in rallying the faithful?

It would be easier to decide whether this news is credible if the book were shorter. Fortunately, much of the text can be discounted on a first reading. Too much space is taken up with the reinterpretation of notorious historical episodes — 'Darwin', 'Lavoisier', 'Galileo' and so on — of which there are already a number of conflicting accounts by sound scholars. Only veterans of these debates would know whether Kitcher's interpretations are reliable. He could surely have exemplified the same theoretical points more convincingly by drawing on the considerable amount of literature on more recent scientific controversies, for which the surrounding cultural environment requires much less explication.

The final chapter, on "The Organization of Cognitive Labor", should also be discounted. It is an algebraic formulation of the social arrangements by which researchers establish and maintain the diversity of effort required to open up an area of research and arrive at a consensus on its description. Not only is Kitcher's formulation incredibly idealized; he also uses a pseudo-quantitative method that was discarded in microeconomics long ago. The problem that Kitcher is tackling is important, but he does not seem to realize that he is essentially dealing with a peculiar type of 'market' system, whose phenomenology is not mathematically deducible from the rational behaviour of a few individual participants. It would have been more helpful if he had simply added his own observations and insights — many of which are interesting — to the existing literature (of which he seems unaware) that takes this point of view.

The basic concept of Kitcher's analysis is what he calls "consensus practice". This is equivalent to one of Thomas Kuhn's more extensive uses of the term 'paradigm' in that it is embodied in individual beliefs and behaviour but transmitted and transformed by communal processes.

Kitcher is realistic in allowing that the notion of a consensus is over-idealized and in distinguishing between veterans and apprentices in the dynamics of change. He wisely concurs with much recent meta-scientific work demonstrating the significant influence of non-cognitive social factors, but equally wisely rejects the extreme doctrines of sects that proclaim the divinity of the sociology of scientific knowledge. He is also refreshingly open to the effects of previous commitments to established authority, instrumental armouries, orthodox methodologies and other historical factors in resisting or facilitating change.

We would all agree that changes in the "cognitive states associated with consensus practice" — that is, in scientific theories — are the substance of scientific progress. For Kitcher these include improvements in the definition of key concepts so as to conform to natural kinds, in understanding the dependencies of phenomena, in accounts of the structure of nature, and in the questions that guide further research. His analysis of 'erotetic' progress in terms of a logical 'tree' of questions designed to escape from a problematic issue is very helpful. For example, it shows how a puzzle (in Kuhn's sense) that seems resolvable by finding a path through such an escape tree, can turn into an anomaly if all such paths are blocked.

Unfortunately, this notion of scientific progress, apt as it is within the context of normal scientific activity, remains epistemologically problematic. Where, we always keep asking, is it progressing to? Kitcher takes the usual escape path towards the supposedly firm ground of a true description of an external reality. Does he succeed in rein-

forcing that treacherous philosophical terrain? He is right to insist that the antirealists should provide a rival picture to "hypothetical fixed nature", and to accept the potential reality of inferred entities, such as electrons, genes and dinosaurs, which are invisible to the naked eye.

Frankly, though, he couldn't hope to win. In the deepest circles of this ancient game, the sceptical card remains the ace of trumps. Yet he could have made much more of two points. In arguing that the general scientific world picture rules out, say, implausible explanations of fossil findings, he is unconsciously appealing to the widely held network model of scientific knowledge, to which he makes no reference. More seriously, he is intellectually snobbish about our 'folk' knowledge of life and nature, forgetting that our touchstone of 'reality' is our completely ineradicable personal sense of the existence of a socially shared life-world. If he were more eclectic in his philosophical reading, he might have noticed that there is a relatively firm escape path for the would-be realist through the open ground between the 'scientific' and 'everyday' domains of knowledge.

Wider reading would also have shown him that he is not the first to propose a socioevolutionary epistemology of this kind. He does see, however, that this requires, above all, an agreed protocol of



Carolina Parakeet by J. J. Audubon (1825). Taken from the superlatively illustrated *Picturing Nature: American Nineteenth-Century Zoological Illustration* by Ann Shelby Blum (Princeton University Press, \$75, £50).

communication and criticism between competing researchers — that is, a generally accepted standard of 'rationality'. Unfortunately, his emphasis on ends-means considerations in the conversations between scientists is too narrow. Surely, the necessary and sufficient criterion of communicative rationality is absence of overt contradiction. Given that as a regulative principle, and given communal mechanisms for extending the scope of publicly 'consensible' knowledge as widely as possible, we may expect the emergence of modern science. Kitcher shows that he is aware of this, through incidental remarks such as "a scientist struggles to eradicate inconsistencies, maintain a unified account of the phenomena . . . and

project regularities", but he still seems unable to rise above the stale orthodoxies of the officially recognized philosophies.

It is not uncommon in science for well-intentioned intellectual energy and imagination to be misdirected and wasted through sheer lack of attention to what other people might have done on the same subject. Kitcher's book will be mined by other scholars for many felicitous observations of the practice of research, but I would be careful not to rely on it as an ally in protecting a reformed 'legend' from its root-and-branch detractors. □

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States of the nations

Margaret Sharp

The Elusive Transformation: Science, Technology, and the Evolution of International Politics. By Eugene B. Skolnikoff. Princeton University Press: 1993. Pp. 322. \$39.50, £30.00.

THE central thesis of Eugene Skolnikoff's thought-provoking book is that science and technology have, over time, had a substantial influence on the evolution of international affairs and that this influence is interactive. The world political system, he suggests, both is affected by and affects developments in science and technology.

This is a huge and ambitious canvas which Skolnikoff divides into three main sections. The first concentrates on the evolution of science and technology systems in the past hundred years, their institutionalization in public and private sectors, and the degree to which these institutions have become self-perpetuating and self-sustaining. The second section explores the effect of developments in science and technology on different aspects of international affairs, including national security, economic and political systems — all of which involve the nation state — and climate change at the global level. Finally, he discusses the implications of these developments for national and international governance and seeks to pull his argument together into a set of general conclusions.

Although the broad relationship that emerges between science and technology and international affairs is a dynamic one, Skolnikoff argues that the process of change is evolutionary rather than revolutionary. In this, he is anxious to refute J. N. Rosenau's hypothesis that developments in science and technology have brought a systemic change to the ordering of international affairs and have led to a

restructuring of relations between nations. In essence his message is that while science and technology have had, and continue to have, a great influence on international affairs, the traditional focus of international relations — on issues such as sovereignty, the nation state and military power — remains unaltered.

This evolutionary message is best illustrated by Skolnikoff's arguments about military developments. Take his discussion of nuclear weapons — an example *par excellence* of the marriage of science and technology to the purposes of the state. He explains how, over time, nuclear weapons "came to exist for the primary purpose of preventing their being used". But, far from making all other forms of military power unnecessary, the nuclear capability has, he argues, tended to encourage the improvement of conventional weapons (to which advances in science and technology have contributed substantially, as exemplified by the 'smart' weapons of the Gulf War). It has also enhanced the role of small states and spawned local arms races. No evidence here, he claims, of a restructuring of the state-centred international system.

Skolnikoff is talented at weaving the argument between science and technology and international developments. In his chapter on economies and politics, he (rightly) singles out the innovative failures of the command economy as a central cause of breakdown, but also shows how this collapse was compounded by advances in information technology, which eroded the ability of the political system to control the flow of information and exposed the credibility of the whole system. In a similar vein, he describes how developments in science and technology have boosted the power of the multinationals, but also led to a reversal of the old arguments of economy of scale and

indeed, on occasion, to a repatriation of activities. These developments have increased international dependencies and put new tools into the hands of government: "The increasing economic importance of technology-intensive products in international trade has altered the traditional determinants of trade relationships, making comparative advantage a supple concept, capable of being created and moulded by national policies to a degree never seen in the past". But again, he concludes: "Significant as these changes are, they have not yet altered, nor are likely to alter, the underlying organising principles of the international political system".

Skolnikoff foresees two main threats to this 'comfortable' conclusion that the nation state reigns supreme. One arises from within science and technology — the increasing use of (and dependence on) large computer-controlled operating systems. In the military sphere there are obvious dangers of false alarms in surveillance systems triggering nuclear attacks; and in the economic and financial sphere, the 1992 devaluations illustrate only too well how pre-programmed systems can orchestrate massive destabilizing swings in the currency markets. Skolnikoff suggests that both may lead to the emergence of new forms of international control and cooperation. In addition, he argues that global warming may present the world with new and overriding problems that can be dealt with only by collective action.

This is not an easy book to read; indeed, I often found myself having to turn back the pages to check the thread of the argument. This complexity seems to occur because Skolnikoff is unsure whether he is addressing the generalist interested in international and business affairs or the academic specializing in international relations. On the whole, the specialist wins through, so that the general is often swamped by the particular.

In pursuing detail, Skolnikoff misses the dynamic central relationship between science and technology and international affairs (the link between technological and political hegemony) that emerges well, for example, from Paul Kennedy's *The Rise and Fall of Great Powers*. Indeed, Skolnikoff quotes Kennedy with approval in his introduction, but his concern to develop the detail of his evolutionary thesis leads him to eschew such a broad sweep. This is a shame, for it would have helped give the book both a focus and an excitement that it lacks. This is not, however, to deny the wealth of its contents. It is full of stimulating and informative ideas that will reward those who persevere to its end. □

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Superconductors and baseballs

Robert J. Cava

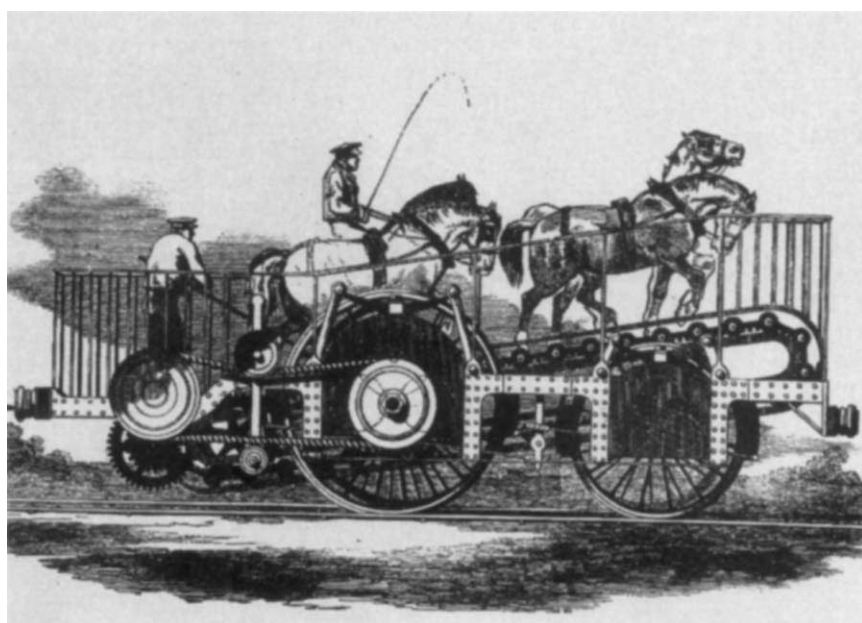
Superconductivity: The Next Revolution?

By Gianfrance Vidali. Cambridge University Press: 1992. Pp. 165. £25, \$49.95 (hbk); £9.95, \$16.95 (pbk).

I CAN still remember the strange recurring dream I was having in late 1986 and early 1987, when we first began working on high- T_c superconductors, of standing at the threshold of a vast, dark room, with the door open barely a crack, sending a narrow shaft of light onto the floor. I would try to open the door further to illuminate the whole room, but it would not move: I could only put my head inside and see what was directly lit, not what was hidden in the darkness. The feeling was one of longing, fear and expectation, for I desperately wanted to see what was inside but was afraid of what it might be and how I might respond. My subconscious was clearly grappling with what many were probably feeling at the time — that we were on the verge of a great moment — and that we would be part of, or at least direct observers of, a historic time in solid-state physics and materials science.

In 1993, after six years of intensive research, the dream (long gone) would show a room swarming with people, every corner being inspected in the cold light of day. Now that the early days are behind us, with the intellectual vigour still fresh in our minds, it is a good time to reflect on how far we have come in high- T_c superconductivity, and to begin to put our generation's contributions to the field into the context of the earlier work. It is in this spirit that Vidali's work is written.

With everything that has been published about superconductivity since 1987, could we possibly need another book on the subject? The answer is yes: one written at a level more detailed and with greater scope than an encyclopaedia or *Scientific American*-style article, more intellectually accessible to interested — but not specialist — readers knowledgeable in physical science than are the professional-level books and articles on the subject. It is this niche that this book has been written to fill, which it does with considerable success. Despite the assertion on the jacket that the book will "fascinate general reader and scientist alike", a knowledge of elementary physics, including some solid-state or atomic physics, would make a big difference to how rewarding the book is. Superconductivity, after all, is a complicated phenomenon, the appreciation of which requires some background and explanation. Vidali does very well in explaining and describing the basic



HORSES for courses — *Impulsoria*, invented in Italy in the mid-nineteenth century, was a locomotive powered by horses and promoted as a means of "extending the advantage of the railway for locations hitherto impracticable". This wood engraving is one of more than a hundred images of science and technology that appear in *Victorian Science and Engineering* by Kenneth Chew and Anthony Wilson. The book reproduces contemporary illustrations from *The Illustrated London News*. Alan Sutton, £12.99, \$25.99 (pbk).

phenomena, concepts and possible technological applications.

The title, preface, jacket and conclusion, however, seem to belong to a different book. Perhaps a straight historical and technical account was not thought to be of sufficient interest to sell the book, resulting in the 'dressing up' of the story as an afterthought. Maybe so, but the book is sufficiently well written and interesting for its intended audience, and the added jazz gets the author into some trouble. The title and some related statements are misleading. Take, for example, the comment on the post-high- T_c world: "suddenly all kinds of applications of superconductivity, from magnetically levitated trains to lossless power lines, became possible". There are, in fact, few in the field today who believe that the high- T_c revolution will change the quality of people's lives the way the semiconductor revolution has. This does not detract from the importance of high- T_c superconductivity. It has truly opened our eyes to part of the physical world we barely knew existed. The body of the book, by contrast, is free from hyperbole. Technical material is presented not in the style of a dry science textbook, subject by subject, but as a historical narrative that leads one along the path of intellectual and technological development in the order in which it unfolded, making the education of the reader virtually painless.

Yet it is in this aspect of the high- T_c story that I find my only real objection to the book. In his essay "The Creation Myths of Cooperstown" on the invention

of baseball, Stephen Jay Gould writes about the conflict between the need for historical accuracy and the strong human craving for 'creation myths' — the belief that truly great inventions must be conceived in creative bursts by god-like figures, rather than through the evolution of ideas involving mistakes, dead ends and the accumulation of both small and large advances. I learned in Gould's essay that Abner Doubleday, the supposed inventor of the great American National Pastime, "didn't know a baseball from a kumquat". I was stunned. Like all gods, the gods of high- T_c have clay feet, and the field has relied for its success a great deal on those who came before and after. Vidali's high- T_c story is less flawed in this regard than its predecessors, but will nevertheless leave the reader with misconceptions. Gould's point, as I read it, is that the popular history of great inventions is not so much recorded as codified — with the need of the human psyche for creation myths playing a pivotal role. Having been witness to the events and now the codification of high- T_c superconductivity, I cannot help wondering whether the chronicle of any great invention has been faithful to the actual process of discovery. □

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■ Kluwer has recently published *Ten Years of Superconductivity: 1980–1990* edited by H. R. Ott, a collection of reprinted articles covering the development of the field. DFL210, \$127, £85.

Recognizing species

Jerry A. Coyne

Evolution and the Recognition Concept of Species: Collected Writings of Hugh E. H. Paterson. Edited by Shane F. McEvey. Johns Hopkins University Press: 1993. Pp. 234. \$32.95, £24.50.

FOR 20 years, Hugh Paterson and his students have waged a *jihad* against one mainstay of evolutionary thought, the biological species concept. Developed by Ernst Mayr and Theodosius Dobzhansky, the concept treats species as interbreeding groups separated from related groups by 'reproductive isolating mechanisms', genetically based barriers to gene exchange such as mate discrimination, ecological preference, and sterility or inviability of hybrids. Paterson and his colleagues contend that this concept is deeply flawed, and, in this collection of 17 published papers, try to replace it with a new view of species.

Their "recognition concept" treats species as "that most inclusive population of individual biparental organisms which share a common fertilization system". In Paterson's view, aspects of the fertilization system most relevant to speciation constitute the "specific-mate recognition system", the set of cues that individuals use to detect conspecific mates (pheromones, courtship rituals and so on). The main difference between the two concepts is that the recognition concept delimits species only through mate recognition, so that other isolating barriers play no part in speciation.

The recognition concept is claimed to remedy several problems with the earlier concept and to aid research on speciation. These claims do not survive close examination. For example, Paterson repeatedly insists that the term 'isolating mechanism' has teleological overtones that mislead supporters of the biological species concept by endorsing the erroneous idea that reproductive barriers are 'mechanisms' directly created by natural selection to protect the adaptive gene complexes of species. (The alternative view, now widely accepted, is that these barriers are usually the accidental by-products of natural selection acting within species.) There was indeed some early confusion about the origin and meaning of reproductive isolation, but just as we do not reject Darwin's views because of the errors and excesses of social darwinists, so we should not reject the biological species concept because it was misinterpreted and over-extended by some of its adherents.

Paterson's second criticism of the

biological species concept is that it is "relational": using it, one recognizes species only by their reproductive isolation from other species. He claims that his recognition concept, by stressing interbreeding *within* a group, defines a species without reference to other species. This is a false distinction. Both concepts are nonrelational when assigning two individuals to the same species (they interbreed); they become relational when assigning individuals to different species (they do not interbreed). Indeed, Paterson admits as much several times: "If the SMRS [specific-mate recognition system] of individuals of a daughter population changes such that they no longer recognize as mates members of the parental population, then a speciation event has occurred".

Does the recognition concept offer any advantages over the biological species concept? The only substantial difference is how they treat forms of reproductive isolation not involved in mate recognition. Populations isolated solely by ecological divergence or hybrid sterility (such as newly formed polyploid plants) are considered species by the biological species concept but not the recognition concept. Paterson argues that taxa isolated from their relatives by intersterility alone could not coexist, for the forms would mate randomly and the rarer would go extinct. If coexistence of genetically isolated forms is essential for speciation, then barriers acting after fertilization do not by themselves delimit species. But this requirement also invalidates the recognition concept—even species isolated by complete mating discrimination cannot coexist unless they also diverge ecologically. Competitive exclusion is common among species with demonstrably different fertilization systems, such as introduced species that outcompete native ones. On balance, the recognition concept is actually subsumed by the biological species concept, in which mate discrimination is considered to be one form of reproductive isolation. I see no advantages in ignoring the other forms.

To be sure, the book is not wholly without value. Its discussion of mate recognition emphasizes an important but neglected aspect of speciation, and Paterson gives a useful assessment of teleological views on speciation, although they are now held by very few. These good arguments, nonetheless, are often mired in inflated rhetoric and marred by out-of-context quotations, questionable assertions that biologists support the biological species concept because of "deep-seated biases inherent in our Western cultural background", excessive repetition (few new ideas surface after the first 50 pages) and tiresome lectures on how to do science (do we really need to hear, for example, that "references provided by authors need

to be checked to see that they say what they purport to say"?).

Finally, the authors' notion that they are overthrowing a major paradigm imbues the book with a revolutionary tone: "Once it is realized that the whole class of isolating mechanisms can be dispensed with entirely, the mind is freed from a set of ancient shackles—true tyrannies of the past—and genetic species can be looked at with fresh eyes". This hyperbole is inappropriate to a "revolution" that proves to be only a noisy skirmish that fails to depose current ideas about speciation. □

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■ Just published is *Species, Species Concepts, and Primate Evolution* edited by W. H. Kimbel and L. B. Martin, the most recent volume in Plenum's *Advances in Primatology* series. The book contains 21 chapters arising from a 1991 symposium. Price, \$138.

■ Species concepts were also the subject of a recent Scientific Correspondence (*Nature* 364, 20; 1 July 1993).

New in paperback

Route 128: Lessons from Boston's High-Tech Community by Susan Rosegrant and David R. Lampe. BasicBooks, \$14. For a review see *Nature* 358, 462 (1992).

The Pill, Pygmy Chimps, and Degas' Horse: The Remarkable Autobiography of the Award-Winning Scientist Who Synthesized the Pill by Carl Djerassi. BasicBooks, \$14. See *Nature* 361, 28 (1993).

Closing Pandora's Box: Arms Races, Arms Control, and the History of the Cold War by Patrick Glynn. BasicBooks, \$17. See *Nature* 360, 386 (1992).

Virus Hunting: AIDS, Cancer, and the Human Retrovirus by Robert Gallo. BasicBooks, \$15.

A Convergence of Lives: Sofia Kovalevskaya, Scientist, Writer, Revolutionary by Ann Hibner Koblitz. Rutgers University Press, \$13.95. See *Nature* 309, 383 (1984).

Butterflies Through Binoculars: A Field Guide to Butterflies in the Boston-New York-Washington Region by Jeffrey Glassberg. Oxford University Press, \$19.95.

The Laboratory of the Mind: Thought Experiments in the Natural Sciences by James Robert Brown. Routledge, £11.99.

Cadillac Desert: The American West and Its Disappearing Water by Marc Reisner. Penguin, \$14.

The Final Forest: The Battle for the Last Great Trees of the Pacific Northwest by William Dietrich. Penguin, \$13.99.

Evolution of the Altaid tectonic collage and Palaeozoic crustal growth in Eurasia

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A new tectonic model, postulating the growth of giant subduction-accretion complexes along a single magmatic arc now found contorted between Siberia and Baltica, shows that Asia grew by 5.3 million square kilometres during the Palaeozoic era. Half of this growth may have occurred by the addition of juvenile crust newly extracted from the mantle, supporting models of considerable continental growth continuing throughout the Phanerozoic eon.

THE orogenic architecture of the Altaids—the late Proterozoic to early Mesozoic orogenic belts surrounding the ancient core region of northern Asia, the Angaran craton (Fig. 1)—has long been recognized as different from that of classical collisional orogens such as the Alps and the Himalayas. The main difference lies in the paucity in the Altaids of extensive ancient gneiss terrains representing former continental fragments, and the molasse-filled foredeeps that generally form between them. The Altaids are formed mainly from material commonly found in present-day subduction-accretion complexes, intruded by vast plutons of mainly magmatic arc origin and covered in places by their volcanic derivatives. Owing to the absence of laterally continuous rock groups and structures that can be used as structural markers to outline the large-scale architecture of the mountain belt, we introduce the 'magmatic front' (that is, the sharply defined oceanward limit of arc magmatism) as a new kind of structural marker to follow the trend-line of the orogen in the Altaids. Doing this allows us to see that the allegedly independent orogens within the Altaid edifice, such as the Ural, Tien Shan, Kazakhstan, Altay/Sayan and Mongol-Okhotsk, may instead have evolved along a single subduction zone, a part of which was located in front of a migratory island arc, called the Kipchak arc. This arc rifted from a combined Baltica/Siberia hinterland in the Cambrian period and became multiply bent (in the manner of an 'orocline') as a consequence of the convergence of these two cratons in the late Palaeozoic. As the Altaid orogen evolved, magmatic arc axes migrated into growing subduction-accretion complexes in front of the Kipchak and the Altay-Mongol arc systems, thereby enlarging the continental crust.

These subduction-accretion complexes added ~5.3 million km² of material to Asia, half of which may be of juvenile origin. This supports interpretations of considerable continental growth during the Phanerozoic, and the Altaid addition to the continental crust may make up as much as half of all the new material added to the continents during the Palaeozoic.

Using magmatic fronts as structural markers may also help in understanding the poorly known Precambrian collages that comprise the cratons, such as the Pan-African system in Arabia. Our reconstruction of Altaid evolution shows that unless detailed geological reconstructions of orogenic collages are attempted, it is unlikely that reconstructing past positions of continental pieces by using only palaeomagnetic, palaeoclimatological and palaeobiogeographical data will have much success.

The Altaid orogenic collage

Suess¹ recognized that Asia had grown peripherally mainly to the west and south of the Angaran nucleus during the Palaeozoic, and he named the resulting orogenic collage the Altaids (Fig. 1). Since then allegedly independent orogens have been distinguished within the Altaids^{2–19}. From east to west, these are

the Mongol-Okhotsk (units 23 and 24 in Fig. 2), Altay/Sayan (units 13 to 22 in Fig. 2), the Kazakhstan 'microcontinent'⁶ (or Kazakhstania²⁰; units 1–12 in Fig. 2, north of 45° N), Tien Shan (units 1–5 and 10 in Fig. 2, south of 45° N), and the Urals (Ural plus unit 1 in Fig. 2)²¹. Even in the first plate-tectonic interpretation of the Urals, Hamilton²² suggested that the Ural, the Kazakhstan uplands and the Altay/western Sayan (Fig. 1) might be parts of a single orogenic system with a sharply bent orocline. Hamilton's view has remained unpopular, despite the growing awareness that the peri-Angaran Palaeozoic orogenic belts are strikingly similar to those farther west^{22–24}.

It has been recognized recently that much of the Altaid collage is formed dominantly from giant Palaeozoic subduction-accretion complexes, surprisingly uniform in timing and style of tectonism, and from the development of subduction-related sedimentation and magmatism, together forming a distinctive orogenic style reminiscent of the circum-Pacific systems called elsewhere 'Turkic-type'^{25–27}. The presence of independent orogenic systems in the Altaids is therefore questionable, especially because the enormous amount of clastic material scraped off into giant accretion complexes must have come from a major continent. If the Kazakhstania microcontinent is 'free floating', this would require a source well away from major continents.

We first review evidence to support the suggestion that all the orogenic belts forming the Altaid collage have evolved dominantly along a single subduction zone that developed during the Cambrian along the eastern margin of a unified Baltico-Siberian continent (Fig. 3a). This hypothesis revives Hamilton's suggestion, albeit with an improved geometry, and also combines the peri-Angaran 'Baykalides' and the 'pre-Uralides' into a single Late Precambrian collision orogen flanking the Baltico-Siberian continent to the west.

Definition of tectonic units in the Altaids

Even in the last century it was well known that the architecture of the Altaids was dominated by 'schists, slates, mafic igneous rocks, and granites'^{1,28}, since interpreted to reflect the dominance of large former subduction-accretion complexes^{25–27}. The monotony of their lithologies, dominated by basalt, chert and turbidites, and the nearly chaotic aspect of their penetrative internal structure make it difficult to find markers in large subduction-accretion complexes by which to outline their large-scale structures. This problem has hindered recognition of the architecture of the Altaids.

To find and map the lateral continuity of large palaeotectonic units, the smallest independently functioning plate-tectonic apparatus, we use magmatic fronts of former arc systems. These fronts are the oceanward limits of magmatism in arcs, and we choose them as structural markers because they tend to be sharp²⁹ and temporally persistent on a scale of tens of millions



FIG. 1 The Altai tectonic collage in its Eurasian setting with the key geographical localities referred to in the text.

of years²⁹, and are continuous along subduction fronts³⁰ (Fig. 2). In our map, we also combine Vendian–Palaeozoic subduction-accretion complexes (only in the extreme east of unit 23.4 is there some Triassic) into a single category, subdivided into three domains according to minimum age of accretion as defined by the age of unconformable forearc sediments and/or intrusive rocks cutting them (Fig. 2). The other domain that we define to help outline the first-order structure consists of pieces of pre-Altaid continental crust. Such pieces invariably turned out to have constituted original arc massifs forming backstops to the accretionary complexes. Within the accretionary complexes, we have noted the accretionary vergence beneath unconformable forearc deposits ('structural vergence' in Fig. 2) and use this as an extra criterion to establish subduction polarity in addition to the facing of magmatic fronts (that is, the direction opposite to the dip direction of subduction zone) and contacts between arc massifs and accretionary wedges.

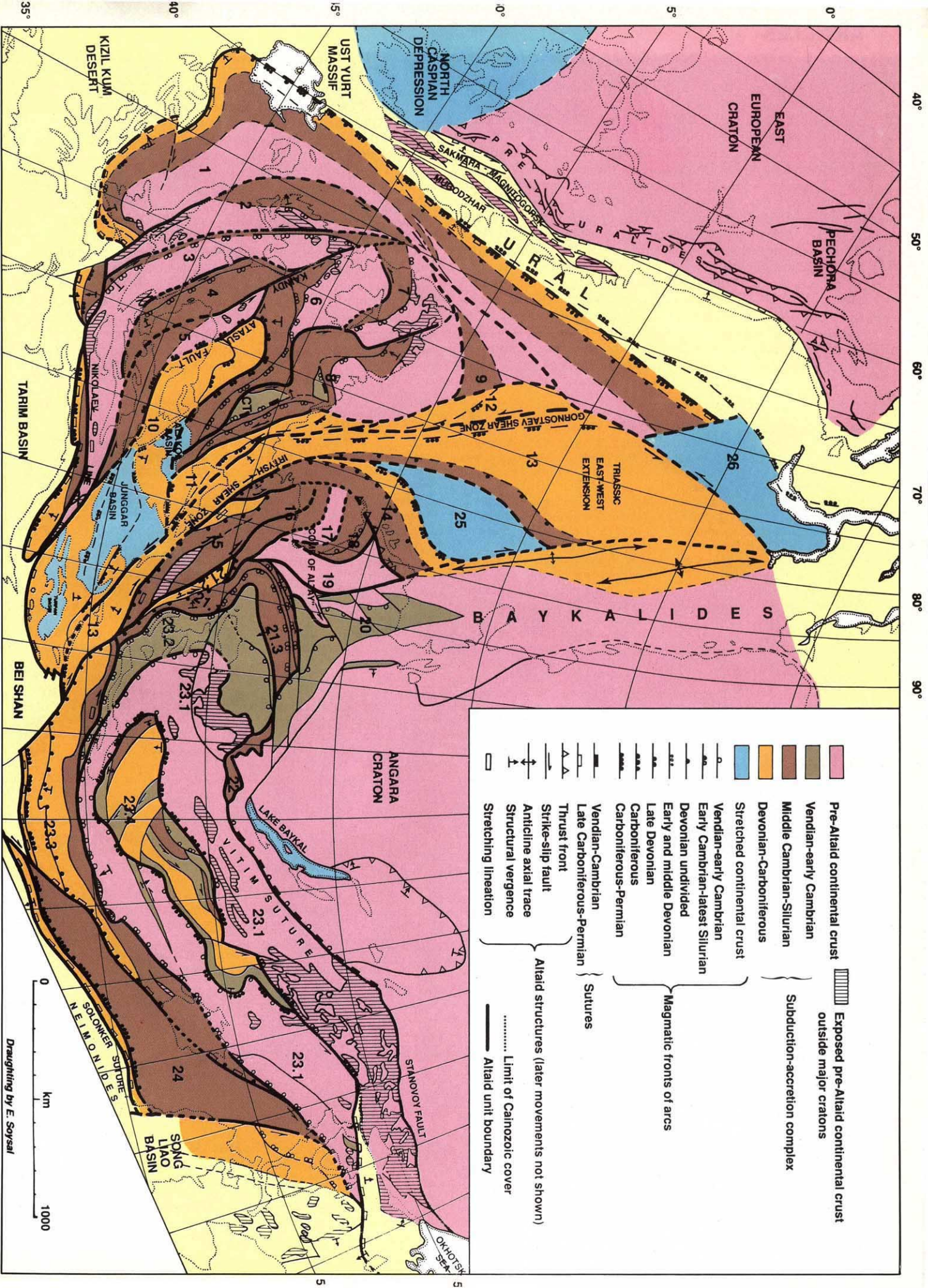
Steep and laterally persistent, straight or gently curvilinear faults along which entire units are truncated were noted as possible strike-slip boundaries and then searched systematically for offsets. Because some former strike-slip faults turned out to have

suffered later significant oroclinal bending and more complicated folding around vertical axes, their identification was an iterative process, proceeding along with palaeotectonic reconstructions.

Regional review

Figure 2 shows the distribution and summarizes the outline stratigraphy and magmatism in the first-order Altaid tectonic units. Most are bounded by large strike-slip faults and many contain a pre-Altaid continental basement of Riphean to locally Vendian

FIG. 2 Generalized tectonic map of the Altaids showing the first-order tectonic units, the pre-Altaid continental fragments, Altaid accretionary complexes and Altaid magmatic fronts. The combined magmatic fronts carry only the symbols representing the earliest and the latest activity. Along the strike-slip faults only the major Altaid displacements are shown, although many of these faults later had motion along them, commonly in an opposite sense; it is these younger motions that are widely mentioned in the literature. Data for structural vergence have been summarized, in addition to the references cited in the text and below, from ref. 64. See Box 1 for a description of the first-order tectonic units.



age, in which pre-Altaid penetrative deformation and metamorphism had ended before the later Vendian. Where data are available, along the large fault boundaries stretching lineations are horizontal and some contain wide mylonite zones, the widest being more than 3 km along the Irtysh shear zone, perhaps the longest-lived strike-slip fault zone in the entire Altaid edifice (Fig. 2, refs 31–33 and B. F. Windley, personal communication), implying very large displacement³⁴. The Vendian in the western Altai (units 1, 2, 5, 7 and 8 in Fig. 2) was a time of rifting characterized by intermediate and mafic volcanic activity, deposition of dominantly non-glaciogenic, mostly marine diamictites and turbidites in rapidly subsiding fault troughs^{35–38}. The onset of arc magmatism and subduction-accretion may have progressed generally from east to west within the Altaid collage during the Vendian–Cambrian interval, although evidence for subduction throughout the entire Altai becomes widespread in the Ordovician. Vendo-Cambrian subduction magmatism and subduction-accretion characterized the margins of units 3, 4, 5,

7, 8, 20, 21.3, 23.1, 23.2, 23.4 and 24. In unit 7 and possibly also in unit 2, it led to ultra-high pressure metamorphism producing metamorphic diamonds and coesite, respectively. The eastern flank of the Kazakhstan orocline is made up of Chingiz-Tarbagatai Vendo-Cambrian tholeiites and Middle Cambrian to Ordovician calc-alkalic volcanics, representing an island arc that originally faced eastward (present geographical orientation)³⁹ within unit 8 (CT in Fig. 2). This is the only fossil arc in the Altai whose place of nucleation with respect to the rest of the orogenic system cannot now be surmised.

In the entire Altai, early Palaeozoic sedimentation outside the pre-Altaid fragments progressed generally from Vendian pelagic ocean floor sediments and distal turbidites through Ordovician and Silurian flysch to Devonian shallow water to terrestrial deposits. After the Devonian, deep water pelagic and flysch sedimentation marched west and south in unit 1, south in unit 3, northeast in unit 5, south and southwest in units 11, 12, 13, 15 and 21.2, northwest in unit 14, and southwest and south in

BOX 1 First-order Altaid units

(1) *Valerianov–Chatkal* (Pre-Altaid continental basement, Altaid accretionary complex and magmatic arc). Pre-Vendian metamorphic basement; Vendian unconformable diamictites forming rift deposits with possible Lower Vendian felsic volcanics; unconformably overlying are Cambrian and Ordovician black slates, limestones and phosphorites; Ordovician to Lower Devonian arc magmatics including both plutons and volcanics; unconformable Middle and Upper Devonian red clastics and Lower Carboniferous limestones; Upper Carboniferous or Permian (locally even possibly Lower Triassic) mainly alkalic felsic magmatism; Ordovician to Devonian fragments of ophiolites, green and bluechists involved in the accretionary complex; uppermost Carboniferous to Lower Permian red clastics overlie the suture to its south and west⁶⁵.

(2) *Baykonur–Beshtash* (Pre-Altaid continental fragment, early Palaeozoic accretionary complex and magmatic arc). Riphean metamorphic clastics and volcanics. In the Makbal complex in the south of the unit these contain quartz pseudomorphs after coesite⁶⁶ which may be of early Palaeozoic age. Locally overlie unconformably by Vendian marine clastics with mafic and felsic volcanics and diamictites forming rift deposits; Cambrian to Middle Ordovician thick limestones interbedded with cherts and phosphorites resting unconformably on older basement; Middle and Upper Ordovician island arc volcanics, coeval tufts in intra-arc and forearc basins; Middle and Upper Devonian red clastics are unconformable on all older rocks; Lower to Upper Carboniferous conformable red clastics and limestones^{65,67}.

(3) *Chu-Terskey* (Pre-Altaid continental fragment, Altaid accretionary complex and magmatic arc). Lower to upper Proterozoic, pre-Vendian metamorphic basement; Vendian–Cambrian ophiolites; Upper Cambrian to Carboniferous are volcanoes and granites; Devonian and Carboniferous clastics in fore- and retro-arc basins; Lower Permian alkaline volcanics and syenites⁶⁵.

(4) *Sarytum* (Pre-Altaid continental basement, Altaid accretionary complex and magmatic arc). Proterozoic metamorphic basement; unconformably overlying Middle Cambrian to Silurian arc volcanics; Lower to Middle Cambrian limestones, cherts and phosphorites; Silurian marine clastics; widespread Devonian granites and calc-alkalic volcanics⁶⁵.

(5) *Atasu-Mointy* (Pre-Altaid continental basement, Altaid accretionary complex and magmatic arc). Riphean metamorphic basement; unconformably overlying Vendian clastics, diamictites and carbonate rocks forming rift fills; Limestones, cherts and phosphorites in the late Vendian to Middle Ordovician; Late Ordovician to Carboniferous granites and island arc volcanics; Devonian and Carboniferous clastics and limestones; Permian syenites and alkalic volcanics⁶⁵.

(6) *Tengiz* (Pre-Altaid continental basement, Altaid accretionary complex, and magmatic arc). Proterozoic metamorphic basement; Vendian marine clastics, cherts and andesites; Ordovician and Silurian flysch and granites; Devonian calc-alkalic volcanics and terrestrial clastics; Carboniferous limestones, coal-bearing deposits, calc-alkalic and alkalic volcanics and granites; Permian alkalic granites and syenites and terrestrial red clastics⁶⁵.

(7) *Kalmyk Kol–Kökhchetav* (Pre-Altaid continental basement, Altaid accretionary complex and magmatic arc). Proterozoic basement, with latest Riphean or early Vendian bimodal volcanics; Vendian diamictites

forming rift fills; early Cambrian ultra-high-pressure rocks including diamonds^{68,69}; Lower to Middle Cambrian basalts, andesitic basalts, keratophyres, tufts, sandstones, cherts and limestones; Lower to Middle Ordovician slates and cherts with intercalated basalts Upper Ordovician conglomerates, sandstones, dacites, andesites and andesitic basalts; Devonian red conglomerates, sandstones, andesites, tufts and granites; Middle Devonian syenites; Lower Carboniferous limestones and coal-bearing clastics⁶⁵.

(8) *Yerementau–Chingiz–Tarbagatai*. Two subunits exist in this unit. One with pre-Altaid continental basement includes the Yerementau fragment and the attached accretionary complex and the other is a tentatively dated Vendian to Middle Ordovician ensimatic island arc that collided with the former in the middle Ordovician. Yerementau basement consists of Riphean and older metamorphic rocks; Lower to Middle Cambrian ophiolites; Middle Cambrian felsic tufts, cherts, slates; Ordovician andesites and basalts, cherts with intercalated basalts and marine clastics; Silurian varicoloured sandstones and siltstones; Late Ordovician granites; Silurian alkalic granites and syenites; Lower Devonian red clastics and magmatic arc volcanics; Middle and Upper Devonian red clastics; Uppermost Devonian and Lower Carboniferous limestones and flyschoid deposits; Middle Carboniferous red clastics^{65,70}. CT is the Vendo-Cambrian, now east-facing Chingiz-Tarbagatai island arc.

(9) *Ishim* (Pre-Altaid continental basement, Altaid arc, Altaid accretionary complex) high-grade Precambrian metamorphic rocks, intruded by both early and late Palaeozoic granites; Ordovician sandstones, slates, cherts and schists of unknown age, unconformably underlying simply deformed Devonian and younger sedimentary and rare volcanic rocks⁷¹.

(10) *Junggar–Balkhash* (Altaid accretionary complex and arc). Upper Cambrian–Ordovician ophiolites; Silurian sandstones and cherty slates; Devonian to Permian clastics, limestones and arc volcanics with granites^{65,72}.

(11) *Zharma–Saur* (Magmatic arc and accretionary complex). Ordovician to Devonian ophiolites; Middle Devonian to Carboniferous island arc volcanics tectonically juxtaposed against marine clastic rocks of the same age; Carboniferous and Permian granitoids⁶.

(12) *Tar–Muromtsev* (Magmatic arc and accretionary wedge: displaced fragment of 11)⁷¹.

(13) *Surgut* (Accretionary wedge overlain by volcanic arc). Most of this is concealed under the West Siberian basin. Extreme southeastern part (Kalba–Narym region): ophiolites, serpentinitic mélange, debris flow deposits, high-pressure/low-temperature schists (545–470 Myr), Lower Silurian to Upper Devonian cherty deep-water carbonate rocks, Middle–Upper Devonian cherts, Upper Devonian to Lower Carboniferous reef limestones and turbidites; main unconformity at the base of the Middle Carboniferous; Upper Devonian to Upper Carboniferous island arc volcanics; intruded by Permian granites. Much enlarged by Triassic east-west stretching^{6,32}.

(14) *Kolyan–Rudny Altay* (Accretionary wedge and volcanic arc). In the extreme south (Rudny Altay) Middle–Upper Devonian volcanic arc atop early Palaeozoic clastics and carbonate rocks intruded by Ordovician, Carboniferous and Permian granitoids; to the west and north, these rocks are replaced by Middle Devonian to Carboniferous turbidites; its

unit 23.3. In unit 23.4 alone, sedimentation became younger centripetally away from unit 23.1 (with respect to present geometry). In all cases magmatic fronts followed the march of deep-water trench sedimentation with a temporal lag of up to ~30 Myr, except in units 1 and 3, where both arc magmatism and migration of deep-water sedimentation were interrupted between the Givetian (late middle Devonian) and the Serpukhovian (later Early Carboniferous). Behind the migrating magmatic fronts, neritic limestones and locally even terrestrial red beds were deposited on shelves, where alkalic magmatism became progressively more widespread after the Silurian.

Trench sedimentation and forearc wedge growth was interrupted after the Silurian in units 2, 3, 4 and 5, and their penetrative southwest-facing fabric became fossilized. In units 3, 4 and 5, the Carboniferous and younger trenches faced away from their earlier counterparts and were located along opposite sides of the units.

As other geologists have emphasized (see for example ref. 2),

the entire Altai system underwent continuous orogenic deformation between the Vendian and the Permian. This is suggested by the numerous local unconformities, gradually shifting magmatic and flysch depositional loci, and the great similarity in style of structures and deformation sequences migrating together with the sedimentary and magmatic axes; it contradicts the hitherto popular model of orogenic episodes affecting the entire orogenic system synchronously. Only in the Permian did high-K anatectic magmatism become essentially 'areal' in the Altai collage, as it began to break up into a mosaic along numerous strike-slip and normal faults and steep thrusts, accompanied by almost entirely terrestrial sedimentation. No Alpine- or Himalayan-type²⁵ crystalline nappe complexes imbricating pre-existing continental crust nor any Indus- or Arosa-type narrow sutures can be recognized within the Altai collage. Continental molasse foredeeps were only constructed after the formation of, and in front of, the Ural, Tien Shan and the Solonker sutures². The flexural ramp valley stages of basins of Junggar-type along

northern part in the Kolyvan range is thrust onto the Anuy-Chuya unit in the Permian^{6,71}.

(15) *Gorny Altay* (Early Palaeozoic accretionary wedge overlain by middle Palaeozoic magmatic arc; farther west in the 'South Altay' middle Palaeozoic accretionary wedge with forearc basin). Vendian-Lower Cambrian ophiolite, HP/LT schists, Middle Cambrian to Lower Ordovician turbidites and debris flows intruded by Ordovician and Devonian granitoids and unconformably overlain by Silurian sandstones, and reef limestones. Later Middle Devonian calc-alkalic volcanics and Upper Devonian non-marine clastics. The 'South Altay' subunit includes Lower Devonian sandstones and shales, Middle Devonian reef limestones, and Middle Devonian-Carboniferous turbidites^{31,32,73}.

(16) *Anuy-Chuya* (Arc, accretionary complex and forearc basin). Cambrian-Lower Ordovician turbidite and debris flow deposits unconformably overlain by Ordovician and Silurian shallow marine sandstones and reef limestones. Along its southwestern margin Ordovician and Lower Silurian turbidites are conformable with older rocks; Lower and Middle Devonian island arc volcanics, Upper Devonian marine and non-marine clastics; Devonian granites^{31,73}.

(17) *Barnaul*: Inferred Precambrian block under Mesozoic-Cenozoic deposits⁷¹.

(18) *Salair* (Accretionary complex and volcanic arc). Cambrian ophiolites, sandstone, shale, limestone tectonically juxtaposed against coeval island arc volcanic rocks; unconformably overlying are Ordovician marine clastics and intermediate composition volcanics; Silurian sandstones and reef limestones; Middle Devonian calc-alkalic volcanics; Upper Devonian marine clastic rocks; all are thrust onto Lower Cambrian sedimentary cover of the Tomsk unit^{6,31}.

(19) *Tomsk* (Pre-Altai continental fragment). Granitic gneisses, amphibolites, schists and thick Vendian-Lower Cambrian terrigenous and carbonate rocks^{6,31}.

(20) *Batenev* (Fragments of the Tomsk unit). Vendian-Lower Cambrian terrigenous and carbonate rocks inferred to lie on Tomsk basement fragments now separated by strike-slip faults of pre-Devonian age⁷³. To the east of the Batenev units are Vendian-Lower Cambrian ophiolites and island arc magmatic rocks marking a suture between the Tomsk microcontinent and the Angara craton. These rocks are all overlain by the Devonian Minussinsk basins of extensional origin containing Devonian alkalic volcanics³⁹.

(21) *Kharkhirin-Western Sayan* (Accretionary wedge and volcanic arc). Includes three subunits formed at the western margin of the Tuva-Mongol unit (21.1) *Kharkhirin sensu stricto* (s.s.) (Middle Cambrian-Lower Ordovician accretionary wedge). Mainly turbidites; unconformably overlain by Ordovician-Silurian andesites, sandstones, mudstones and limestones deposited in an intra-arc setting behind a front migrated west from the Tuva-Mongol unit. Silurian and Devonian granitoids are stitching plutons between the Kharkhirin and Tuva-Mongol units^{32,33}. (21.2) *Delyun-Sagsai* (Accretionary wedge and forearc). Middle and Upper Devonian sandstones, shales, subordinate cherts; considered a fault-separated part of the Kharkhirin s.s.⁷³ (and B. F. Windley, personal communication, 1993). (21.3) *Western Sayan* (Accretionary complex and island arc). Vendian-Lower Cambrian ophiolites, HP/LT schists, ophiolitic mélanges, Cambrian-Silurian turbidites and

subordinate cherts, reef limestones; all unconformably overlain by Upper Silurian coarse clastics and intruded by granites⁶.

(22) *Oka-Jedinsk* (Volcanic arc and accretionary complex). A fault-bounded wedge of probable Vendian to early Cambrian ophiolites and debris flow deposits, Ordovician-Silurian cherts, flysch, and limestones; possible Cambro-Silurian island-arc volcanic rocks. Both assemblages thrust onto the Tuva-Mongol unit^{6,74,75}.

(23) *Tuva-Mongol sensu lato* (s.l.) This major unit has a number of subunits little displaced with respect to the pre-Altai continental core of the unit. These subunits are: (23.1) *Tuva-Mongol s.s.* (Vendian-Permian magmatic arc on pre-Altai continental basement): Early Precambrian to Riphean metamorphic rocks, locally separated along later strike-slip faults by bands of Vendian-Lower Cambrian ophiolites; unconformably overlain by Vendian-Cambrian shelf carbonates; coeval calc-alkalic volcanics frame its margins; early, middle and late Palaeozoic granites, and subordinate Devonian and Permian syenites; middle and late Palaeozoic clastic rocks in diverse types of superimposed basins; Devonian calc-alkalic and abundant Permian calc-alkalic and alkalic volcanics^{33,76}. (23.2) *Ozernaya* (Tuva-Mongol magmatic arc which migrated onto an accretionary complex). Vendian-Lower Cambrian ophiolites and ophiolitic mélange; Lower Cambrian clastic rocks, limestones, cherts, debris flow deposits, intruded by Cambrian and Devonian granitoids^{31,72,77,78}. (23.3) *South Mongolian* (Accretionary wedge which grew to the south of the Tuva-Mongol s.s. unit from the Ordovician to the early Carboniferous). Ophiolites, Silurian-Devonian cherts, limestones, Ordovician to Lower Carboniferous marine clastic rocks, Upper Silurian to Lower Carboniferous andesites, dacites and rhyolites, Lower Carboniferous debris flow deposits; Devonian reef limestones unconformable on lower Palaeozoic sandstones and shales; all of the above were imbricated with south vergence and age of calc-alkalic volcanics become younger in the same direction; all of the above are unconformably overlain by mid-Carboniferous to Permian volcanic and sedimentary rocks⁷⁹⁻⁸¹. (23.4) *Khangai-Khantey* (Accretionary wedge and magmatic arc). Vendian-Lower Cambrian ophiolites, Lower Palaeozoic to Carboniferous turbidites, mafic and intermediate volcanic rocks, tuffs, subordinate cherts; all of the above were intruded by Permian, Triassic and Jurassic granitic rocks³³.

(24) *South Gobi* (Magmatic arc on pre-Altai continental basement). Precambrian granitic gneisses, Riphean to Lower Cambrian clastic and calc-alkalic volcanic rocks, Ordovician to Lower Devonian turbidites, Lower Devonian calc-alkalic volcanics ranging from andesites to rhyolites; early Palaeozoic granites; along its southern rim Carboniferous flysch with southerly imbrication; all unconformably overlain by Upper Carboniferous and Permian calc-alkalic volcanics⁸²⁻⁸⁴.

(25) *Nurul* (Permo-Mesozoic basin overlying stretched continental crust). Silurian to Middle Carboniferous deformed marine clastic and carbonate rocks intruded by late Palaeozoic alkalic basaltic sills and dykes. Total thickness of crust is reduced to 36 km (refs 49, 71) compared with more than 40 km in surrounding areas.

(26) *Nadim* (Permo-Mesozoic basin overlying stretched continental crust). One of the deepest parts of the West Siberian basin, similar to the Nurul basin^{49,71}.

the southern Altaids originated only after the onset of Cimmeride collisions to the south in the late Triassic⁴⁰.

Tectonic evolution

In the following description, all orientations refer to the reconstructed geographies shown in Fig. 3. The most important constraint for the initial conditions of the Altaid evolution is the interpretation that Baltica (Vendian–Palaeozoic nuclear Europe including the east European craton) and Siberia (Vendian–Palaeozoic nuclear Asia including the Angara craton) may have been attached to one another along their present northern boundaries during the Vendian. Palaeomagnetic data^{41,42} allow us to place the two cratons as shown in Fig. 3A. Late Vendian rifting between Baltica and Siberia is indicated on the European margin by the 600-Myr dykes in both Finnmark⁴³ and the northern Kola Peninsula⁴⁴, and by extensive Vendian diamictite deposition and volcanism in tectonically active basins⁴⁵. Their Siberian counterpart may be located in the poorly dated (possibly latest Riphean–Vendian) rift fills of the Taymyr peninsula, whose ages have recently been revised to become younger and thus closer to the European rocks⁵.

The combined Baltica/Siberia had what appears to have been an active eastern margin underlain by the Riphean/Vendian pre-Uralide collisional orogen to the east of Baltica⁴⁶ and by the Baykalide collisional orogen to the east and north of the Angaran craton of Siberia⁵. The Baykalide collision of the Tuva–Mongol (unit 23.1), Tomsk (unit 19) and Barnaul (unit 17) microcontinents with the Angara craton may in fact have triggered the subduction outboard of the collided fragments, although to the east and northeast of unit 23.1 Vendian subduction continued into the Palaeozoic, directly linking the Baykalide and Altaid evolution.

Already during the Vendian, segments of this marginal orogen began to rift from the combined Baltica/Siberia. Active rifting is documented in units 1, 2, 3, 5 and 7. The conjugate Baltica/Siberia margin contains similar evidence in the Northern Ural^{5,47}. In addition, a line of 'Riphean to Palaeozoic rifts' with Vendian–Cambrian fill characterizes the present western boundary of the Baykalides in the basement of the west Siberian basin^{48,49}. This rifting event occurred later southwards towards the present-day southern Ural, where it occurred at the Cambro–Ordovician transition⁶.

We term the fragment thus rifted off Baltica/Siberia the Kipchak arc, after the former aboriginal language group spoken in the area where its fragments are now found. Until the Devonian it bowed away from the two cratons with a 'free end' possibly attached to a transform fault at its southern terminus (Fig. 3B), while at its northern end it probably remained attached or close to Siberia, where the early Palaeozoic accretionary complex in units 8, 15 and 16 is larger than in other parts of the arc (Fig. 3B). The accretionary complex of the Kipchak arc became progressively smaller towards the southwest away from its main source in Siberia and may have resembled the present Aleutian accretionary complex⁵⁰. The ocean that opened behind the Kipchak Arc is herein called the Khanty–Mansi ocean after the aboriginal population of northern West Siberian lowlands. At this time the Sakmara/Magnitogorsk marginal sea in the southern Urals was expanding, possibly behind the migrating Mugodzhaz arc⁵¹.

By early Devonian time (Fig. 3C), growth of subduction-accretion complexes in front of units 1–5, ceased. We interpret this as their being laterally stacked along their bounding strike-slip faults as shown in Fig. 3C. The cause of this strike-slip stacking may have been the collision of the southern end of the Kipchak arc with the Mugodzhaz arc in the southern Ural, as suggested by the sudden influx there of thick Early Devonian clastics and a concurrent drop in subduction-related magmatism⁵¹. A Devonian subduction-accretion wedge, by contrast, began growing to the north of units 6, 7 and 8, and the Devonian magmatic front migrating onto them turned them into

new 'arc massifs' (Fig. 2).

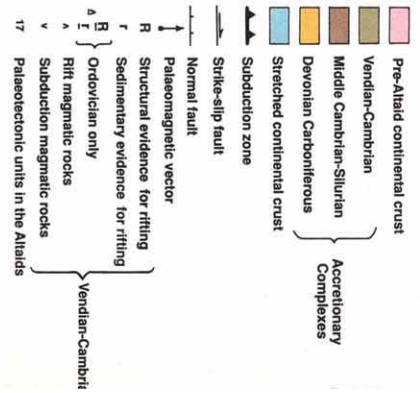
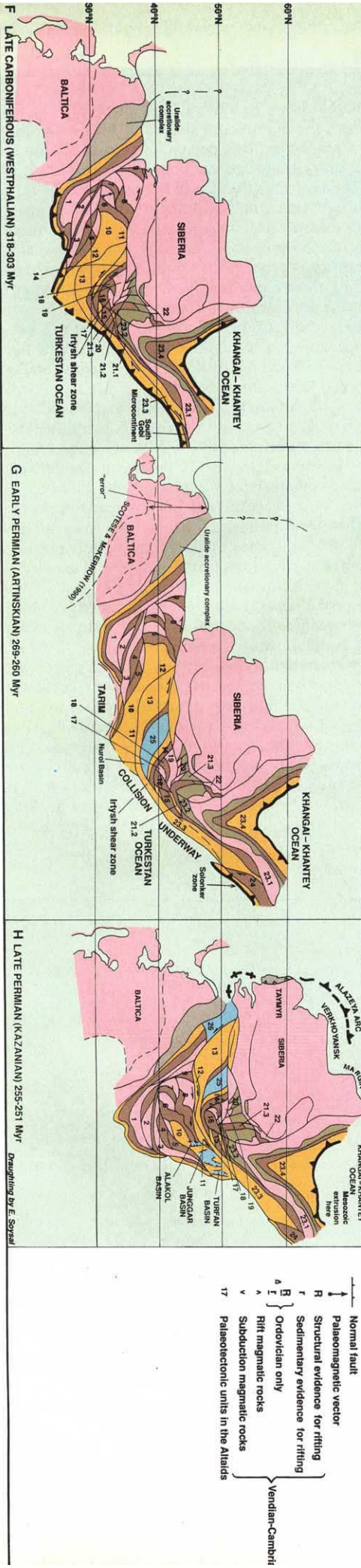
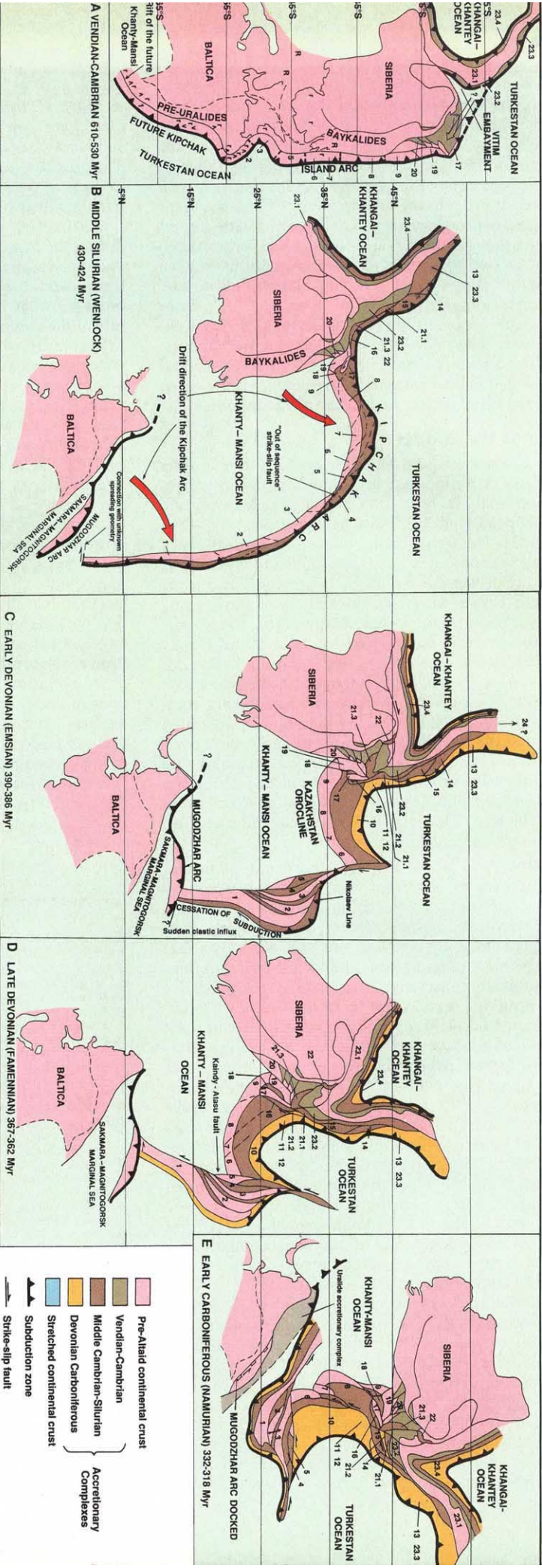
Oblique southwesterly subduction at this time was going on all along southern Mongolia and the Altay mountains. The oceanic Vitim embayment (Fig. 3A and B), lined by the western Sayan arc (unit 21.3), had closed by tightening of the gulf and had pinched the western Sayan arc by the Late Silurian (Fig. 3B and C). This tightening was probably accomplished by an eastward motion of unit 23.1 that entrained the ophiolites associated with the western Sayan arc along it and gave rise to the Oka–Jedinsk zone (unit 22; Fig. 3C). The Anuy–Chuya fragment (unit 16) of Cambro–Ordovician accretionary complex bypassed the western Sayan pinching and was emplaced against the Barnaul unit (number 17) by right-lateral strike-slip faulting. This led to further strike-slip stacking within the 'Comb of Altay' (Fig. 2) and generated the alkalic volcanism in the Minusinsk basins³¹.

By late Devonian time (Fig. 3D), the present apex of the Kazakhstan orocline had moved left-laterally along the Kaindy Atasu fault (see Fig. 2) relative to its present southwestern flank, leading to arc magmatism along the present northeastern front of the Atusu–Mointy fragment (unit 5). It may also have begun to be internally disrupted by strike-slip faults (now) parallel with the Kaindy Atasu fault, as suggested by the Middle Devonian alkaline intrusives at least along the boundary between the future units 7 and 8 (ref. 52) (Fig. 2). By this time, a substantial subduction-accretion complex had grown inside the Kazakhstan orocline, into which the magmatic front migrated (Fig. 2). Within the older subduction-accretion complex, across which the magmatic front had moved, the intrusion of high-K granites in the retroarc area and the deposition of shallow water sedimentary rocks in the forearc region suggest that a continental crust of near-normal thickness was most probably generated here by subduction-accretion and arc magmatism²⁶.

In the Early Carboniferous (Fig. 3E), the approach of Baltica to Siberia at the expense of the intervening Sakmara–Magnitogorsk, Khanty–Mansi and the Turkestanian oceans led to transpressional slip of the tip to the Kipchak arc to the north and caused subduction to resume under the segment now represented by units 1 and 2. Ongoing oblique subduction along southern Mongolia and the Altay emplaced the Gorny Altay (unit 15) against the Delyun–Saksai Devonian accretionary wedge (unit 21.2) and thus terminated its growth. By this time the apex of the Kazakhstan orocline had probably begun tightening by extensive internal strike-slip faulting separating the Tengiz, Kalmyk Kol–Kökchetav and the Yerementau–Chingiz–Tarbagatai (units 6, 7 and 8, respectively) from one another, because the faults separating these fragments all affect Carboniferous rocks (some with later smaller right-lateral slip owing to north–south shortening⁵³, not shown in Fig. 3).

By late Carboniferous time, Baltica had begun shearing with respect to Siberia in a right-lateral sense (Fig. 3F). This shear was combined with convergence and thus both tightened the entire Kazakhstan orocline plus unit 1 and transported it westward with respect to Siberia, this continuing the right-lateral shear along the Altay–South Mongolian area. Both the Rudny Altay–Kolyvan unit (14) and the combined Zharmas–Saur, Tar–Muromtsev, Surgut units (11, 12 and 13, respectively) moved west with respect to the 'Comb of Altay'. This movement continued into the Early Permian (Fig. 3G) pulling apart the Nurol basin (unit 25) and leading to alkaline magmatism in its basement.

FIG. 3 Schematic and simplified palaeotectonic sketch maps showing the evolution of the Altaids. Positions of continents were modified from refs 20, 41, 42. Palaeomagnetic vectors shown were averaged from determinations 08080, 08081, 08082 and 08110 in ref. 85. In G the $\pm 10^\circ$ 'error' is with respect to the position given in ref. 20 and may have resulted from shortening in the Urals, shortening in Kazakhstan or palaeomagnetic measurement error.



The last act of the Altaid evolution during the Palaeozoic was a reversal of the shear sense between Baltica and Siberia (Fig. 3H). This reversal was accompanied by extension across the future west Siberian basin as Baltica and its appendage west of the Gornostaev shear zone (Fig. 2) swung south and east with respect to Siberia. It brought the arc fragments of Zharmasaur (unit 11) and Tar-Muromtsev (unit 12) eastward with respect to Siberia, and juxtaposed them against the Carboniferous flysch deposits of unit 13 (ref. 6). A part of this transtensional regime was responsible for rifting open the Nadim, Alakol, Junggar and the Turfan extensional basins, before the latter three fell prey to late Triassic compression resulting from the Cimmeride collisions far to the east. The same kinematics led to collision in the Taymyr and to ongoing subduction along the Alazeya arc behind the Verkhoyansk belt in northeastern Siberia and may have been responsible for the onset of the Tunguska trap eruptions.

Implications of the model

The Palaeozoic evolution of the Altaid orogenic collage in Asia records the origin, evolution and disruption mainly by strike-slip faulting, and final amalgamation into a complex orogenic collage, of the Kipchak and the Tuva-Mongol/Comb of Altay arcs (Figs 2 and 3). The initial rifting by back-arc spreading of the Kipchak Arc was probably similar in scale and style to the opening of the ocean basins west of the boundary between the New Zealand and Tonga-Kermadec-Melanesian plates⁵⁴. Long non-subduction segments that existed mainly during the Middle Palaeozoic in the segment represented by units 1 and 3 may have been similar to the north Fiji basin segment of the peri-Australian arc systems⁵⁵. Our Fig. 3B depicts the Kipchak as a single, simple arc. Although the present observations are compatible with this, comparison with the active analogue cited above suggests that the tectonic picture was probably more complicated, and the arc may well have been fragmented along its strike. The necessity of introducing 'out-of-sequence' strike-slip faults during the stacking of the western flank of the Kazakhstan orocline before the Early Devonian (Fig. 3B) may indicate complications not foreseen by our simple model.

Continental growth. The use of magmatic fronts of former arcs as structural markers, combined with arc massif/accretionary wedge contacts, has proved fruitful in deciphering the gross architecture of a vast and complicated region stretching from the Ural to eastern Asia. By contrast, our analysis shows that ophiolites may be misleading as structural markers in continuously growing subduction-accretion complexes across which arc magmatic axes prograde oceanwards in episodic jumps. Ophiolites in such terrains occur in a haphazard fashion as accreted slivers and fragments and carry no significance as structural markers to delineate former palaeotectonic entities as they do in Himalayan or Alpine-type collision orogens. Contrary to conventional wisdom^{18,19,21,56-58}, discrete arc magmatic fronts in the Altai do not represent former individual island arcs, although they are separated by terrains bearing abundant ophiolitic fragments. Where accretionary complexes dominate orogenic architecture, for example in the Pan-African terrains of northeast Africa and Arabia, it is important for our understanding of the growth of continental crust to distinguish individual arcs from migrating arc axes within a growing accretionary complex of a single arc, because the former interpretation would produce a larger number of sutures than actually exists.

This tectonic evolution was similar to that which characterized the tectonic evolution along the North American Cordillera, and especially in Alaska, during the Mesozoic and early Cenozoic. An important aspect of it was the generation of immense subduction-accretion complexes along the Tuva-Mongol/Comb of Altay and the Kipchak arc front. The total area now occupied by these complexes within the Altai is some 5.3 million km², or about one-ninth of the entire surface of Asia. During the ~350 million years of Altaid evolution, an area slightly larger than two soccer fields thus was added to Asia every year. Howell

& Murray⁵⁹ estimated that 1.30 km³ of sialic material is generated every year from the mantle and some 1.70 km³ is fed into subduction zones from terrigenous, pelagic and biogenic sources. Thus, of the total accreted to the continents, about 45% is of juvenile and 55% of exogenically recycled sources. If this ratio has remained constant since the beginning of the Phanerozoic, ~0.205 km³ of juvenile material must have been added annually during the Palaeozoic to the continental crust along Altaid subduction systems supporting models of significant growth of continental crust during the Phanerozoic⁶⁰. Dewey and Windley⁶¹ estimated that during the Mesozoic-Cenozoic, the net growth rate of the continental crust has been 0.429 km³ yr⁻¹. If this were so also for the Palaeozoic, it suggests that the Altaid subduction systems may have contributed nearly 48% of the Earth's total of new continental crust during the Palaeozoic.

Our estimate of ~2.5 million km² of juvenile matter added to Asia in ~350 Myr makes Coney's⁶² figure of 7.23 million km² added to North America during only 165 Myr since the middle Jurassic, based on the supposed extent of the oceanic and juvenile arc material, rather surprising. Owing to the absence of continental glaciers, Mesozoic sea level was higher than that in the Palaeozoic⁶³ and therefore continental denudation was probably slower. This would probably have made the turbidite input into the trenches, and thus subduction-accretion slower. The alleged significantly larger growth rate of North America in a time span less than half that of the Altaid evolution might therefore imply that the volume of oceanic subduction-accretion material in the North American Cordillera was overestimated.

Palaeotectonic reconstructions. Finally, our reconstruction of the evolution of the Altaid collage has shown that reconstructions of major continental units during the Palaeozoic based on the detailed geological structure of present tectonic collages is likely to lead to a more refined picture than those attempted on the basis of palaeomagnetic, biogeographic and palaeoclimate data alone (for example, refs 20 and 41). In particular, there is the possibility of recognizing, by combined structural geology and stratigraphy, very-large-scale latitudinal strike-slip motion. □

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Normal and oncogenic p21^{ras} proteins bind to the amino-terminal regulatory domain of c-Raf-1

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In higher eukaryotes, the Ras and Raf-1 proto-oncoproteins transduce growth and differentiation signals initiated by tyrosine kinases. The Ras polypeptide and the amino-terminal regulatory domain of Raf-1(residues 1–257) are shown to interact, directly *in vitro* and in a yeast expression system. Raf-1(1–257) binds GTP-Ras in preference to GDP-Ras, and inhibits Ras-GAP activity. Mutations in and around the Ras effector domain impair Ras binding to Raf-1(1–257) and Ras transforming activity in parallel.

IN higher eukaryotes, the *ras* and *raf-1* proto-oncogenes are indispensable sequential elements in the transduction of growth and differentiation signals initiated by receptor and non-receptor tyrosine kinases^{1,2}. Whereas the element regulated directly by yeast Ras was identified early on as adenyl cyclase³, the identities of the immediate downstream targets for Ras in mammalian cells are not yet known¹. Recent work, however, indicates that the activation state of a set of cytoplasmic protein Ser/Thr kinases, including c-Raf-1, MEK, MAP kinase (ERK-1/ERK-2) and Rsk, is rapidly and perhaps directly regulated by the Ras protein. Introduction of active Ras polypeptides into *Xenopus* oocytes^{4–6} or mammalian cells^{6–8} rapidly activates MAP kinase; dominant negative *ras* mutants block activation of Raf-1 (refs 8–10) MAP kinase^{7–9} and Rsk (ref. 8) by receptor tyrosine kinases. Several reports indicate that addition of Ras polypeptide to cell-free extracts prepared from mature *Xenopus* oocytes activates the endogenous MAP kinase^{5,11,12}; such activation requires a Ras polypeptide that has been fully processed at its carboxy terminus and is in the GTP-bound state¹². A mutation in the Ras effector domain (D38→E) which abolishes Ras transforming activity prevents *Xenopus* MAP kinase activation⁴. Considerable evidence indicates that these four cytoplasmic protein (Ser/Thr) kinases are arranged in a linear cascade; Rsk is phosphorylated and activated by p42/p44 MAP kinases^{13–15}, which are in turn activated by MEK^{16,19}. c-Raf-1 phosphorylates and activates MEK *in vitro*^{20–22}; moreover, a *Drosophila* gene encoding a MEK-like kinase has been isolated as a suppressor of *Drosophila raf-1* loss-of-function mutations²³. Among these four Ras-regulated cytoplasmic Ser/Thr kinases, c-Raf-1 is the element situated most proximal to the signal generated by Ras.

In NIH 3T3 cells the essential role of c-Raf-1 in the transduction of proliferative signals initiated by receptor tyrosine kinases and transmitted through c-Ras is demonstrated by the ability of antisense *c-raf-1* RNA or dominant negative *c-raf-1* mutants to

block the DNA synthesis and growth stimulated by serum, the phorbol ester TPA or *ras* oncogenes²⁴. Further evidence for the central role of Raf-1 as a downstream effector of Ras is the ability of *v-raf* expression to overcome the blockade to proliferation that prevails in *ras* revertant lines²⁵ or which is induced by microinjection of inhibitory anti-Ras antibodies²⁶. Although the evidence showing that Ras is necessary for the growth-factor-induced activation of c-Raf-1 *in situ* is strong, the mechanism underlying Ras activation of Raf-1 kinase is unclear. In most cells, growth-factor-induced Raf-1 activation is accompanied by multisite (Ser/Thr) phosphorylation of the Raf-1 polypeptide through a kinase-C-independent pathway (reviewed in ref. 2); treatment of the activated c-Raf-1 polypeptide *in vitro* with the Ser/Thr-specific phosphatase-1 causes deactivation of Raf-1 kinase²⁷. Many (but not all) of the tryptic ³²P-labelled peptides phosphorylated on Raf-1 *in situ* during growth factor stimulation of NIH 3T3 cells comigrate with ³²P-peptides generated by phosphorylation of Raf-1 *in vitro* by p42/p44 MAP kinase; such *in vitro* MAP kinase-catalysed c-Raf-1 phosphorylation, however, does not activate c-Raf-1 kinase activity²⁸.

Raf-1 contains, in addition to the conserved carboxy-terminal kinase catalytic domain, two other highly conserved regions (CR1 and CR2) in the amino-terminal half of the molecule². The CR1 and CR2 segments seem to be regulatory, inasmuch as mutations or insertions in these regions and their deletion activate Raf-1 transforming and transactivating activities^{29,30}. Moreover, the ability of a kinase-inactive c-Raf-1 polypeptide, created by mutation of the ATP-binding site (K375→W), to act as a dominant inhibitor of Ras-dependent gene transcription³¹ and proliferation²⁴ is reproduced fully by c-Raf residues 1–257, a segment that contains CR1 but not CR2. The CR1 domain contains a cysteine finger (C₁₅₂X₂CX₉CX₂C₁₆₈) whose integrity is crucial for normal regulation of Raf-1 function, and for the ability of the amino-terminal domain (1–257) to act as a dominant inhibitor of *ras* effects. Disruption of the cysteine finger by mutation (C168S) produces a Raf variant whose basal function

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(assayed by cotransfection with an API/Ets-driven CAT reporter) is partially activated but no longer susceptible to further activation by Ras, or to inhibition by dominant inhibitory *ras* mutants. In a reciprocal fashion, the C168S mutation greatly attenuates the ability of the Raf-1 amino-terminal domain to act as an inhibitor of Ras-dependent responses³¹. These observations indicate that the c-Raf CR1 domain is crucial for Ras regulation of Raf kinase activity, perhaps mediated through the direct interaction of the c-Raf CR1 domain with a Ras-dependent signal, accompanied by (Ser/Thr) phosphorylation of the Raf polypeptide, thereby stabilizing an active state.

Ras and Raf interact directly *in vitro*

Based on these considerations, we determined whether the Ras-dependent signal that interacts with the c-Raf-1 amino-terminal regulatory domain was the active Ras polypeptide itself. Glutathione-S-transferase (GST) fusion proteins encoding c-Raf(1–257) and mutant c-Raf(1–257, with C168→S), and GST alone, were added at equal concentrations, to varying amounts of GTP- γ S-loaded V12G-Ha-Ras; no binding of GTP- γ S-V12G-Ha-Ras to GST alone is detected (Fig. 1a, lane 1). By contrast, a dose-dependent binding of V12G-Ha-Ras to GST-Raf(1–257) is seen (Fig. 1a, lanes 2–4). GTP- γ S-V12G-Ha-Ras also binds to GST-Raf(1–257C168S) (Fig. 1a, lanes 5–7; Fig. 1b), but for any concentration of Ras, much less Ras is retained by equal amounts of mutant Raf(1–257C168S) compared with wild-type Raf(1–257) (Fig. 1a; compare lanes 2–4 to lanes 5–7; Fig. 1b).

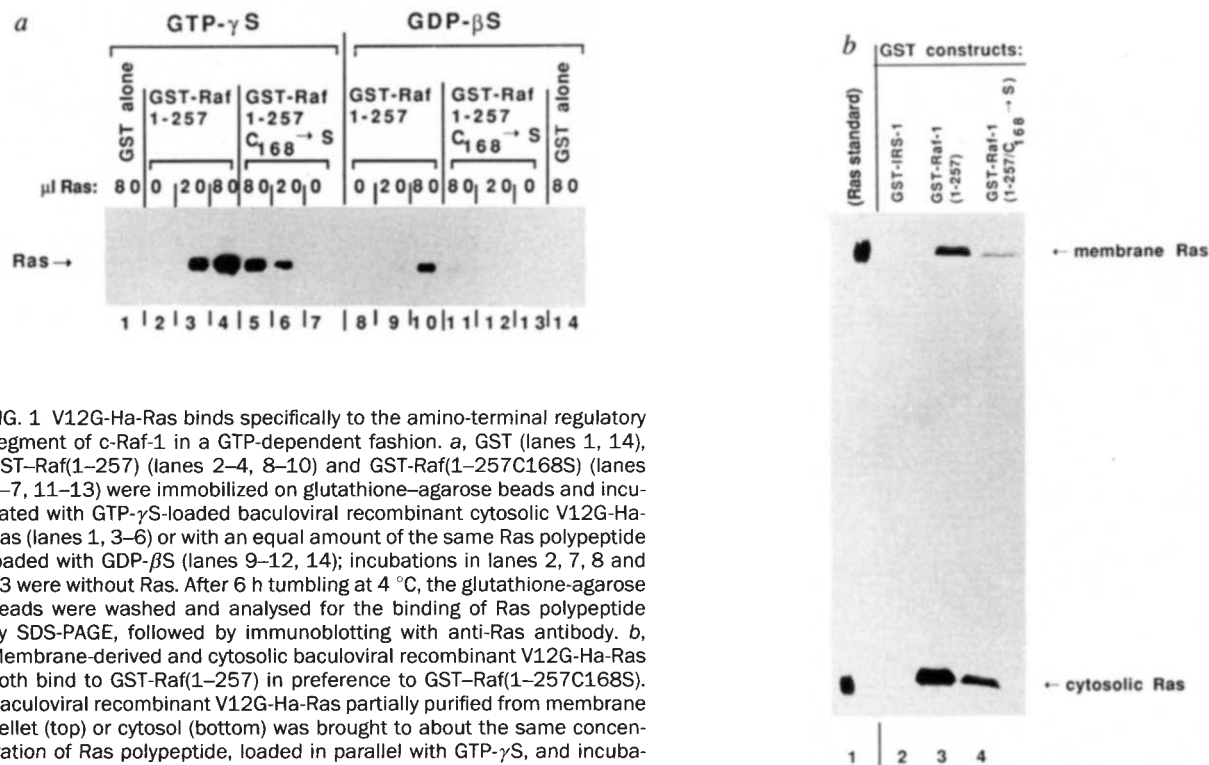


FIG. 1 V12G-Ha-Ras binds specifically to the amino-terminal regulatory segment of c-Raf-1 in a GTP-dependent fashion. **a**, GST (lanes 1, 14), GST-Raf(1–257) (lanes 2–4, 8–10) and GST-Raf(1–257C168S) (lanes 5–7, 11–13) were immobilized on glutathione-agarose beads and incubated with GTP- γ S-loaded baculoviral recombinant cytosolic V12G-Ha-Ras (lanes 1, 3–6) or with an equal amount of the same Ras polypeptide loaded with GDP- β S (lanes 9–12, 14); incubations in lanes 2, 7, 8 and 13 were without Ras. After 6 h tumbling at 4 °C, the glutathione-agarose beads were washed and analysed for the binding of Ras polypeptide by SDS-PAGE, followed by immunoblotting with anti-Ras antibody. **b**, Membrane-derived and cytosolic baculoviral recombinant V12G-Ha-Ras both bind to GST-Raf(1–257) in preference to GST-Raf(1–257C168S). Baculoviral recombinant V12G-Ha-Ras partially purified from membrane pellet (top) or cytosol (bottom) was brought to about the same concentration of Ras polypeptide, loaded in parallel with GTP- γ S, and incubated with GST-IRS-1 fusion protein, GST-Raf(1–257), or GST-Raf(1–257C168S) immobilized on glutathione-agarose. After 6 h tumbling at 4 °C, the beads were washed and analysed for the binding of Ras polypeptide as in **a**.

METHODS. *Spodoptera frugiperda* (Sf9) cells grown in monolayer were infected at 5 $\times$ multiplicity with a recombinant baculovirus encoding V12G-Ha-Ras. 65–72 h post-infection, cells were collected as in ref. 46, sonicated, and centrifuged at 100,000g for 1 h. The Ras polypeptides in the membrane pellet and cytosol were each purified separately by Mono Q anion-exchange chromatography⁴⁶ using 30 mM *n*-octylglucoside in both purifications. The peak of [³⁵S]GTP- γ S binding corresponded to the Ras polypeptide detected by immunoblot; both preparations (stored at –80 °C in 20 mM Tris-Cl, pH 8, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 30 mM *n*-octylglucoside and ~0.25 M NaCl) were ~10% pure. Nucleotide loading of Ras was by dilution to the desired polypeptide concentration and addition to an equal volume of 2 $\times$ loading buffer (0.1 M Tris-Cl, pH 7.5, 15 mM EDTA, 1 mg ml^{–1} BSA, 2 mM DTT, 1 mM GTP- γ S or GDP- β S). After 15 min at 37 °C, MgCl₂ was added to 12.5 mM and the Ras polypeptide placed on ice. Charging of Ras with [³⁵S]-labelled guanyl nucleotides under these conditions indicated that a stoichiometry near 1.0 was achieved for both GTP- γ S and GDP- β S. A mock GTP/GDP charging reaction contained all components except that Ras was replaced by Ras storage buffer. The DNA sequences encoding c-Raf-1 amino-acid residues 1–257 were generated by polymerase chain reaction (PCR), using the plasmids RSV-C4 and RSV-PM17 as templates³¹. These encode the wild type and C168S mutant sequences, respectively. The cDNAs were inserted into pGEX-KG (ref. 47) at the *Eco*R1 and *Sal*I sites, and sequenced to confirm the frame and the mutation. The GST-Raf fusion proteins, and the GST polypeptide

were purified on glutathione-agarose and eluted with glutathione (12.5 mM) in buffer A (50 mM Tris-Cl pH 7.5, 0.15 M NaCl, 1 mM DTT, 1.0% Triton X100); glutathione was removed by dialysis against buffer A. On staining after SDS-PAGE, GST-Raf(1–257) and GST-Raf(1–257C168S) were indistinguishable, with ~50% of the recombinant polypeptides recovered as full-length fusion protein. The purified GST and GST-Raf fusion proteins were each incubated with glutathione-agarose at 7 mg polypeptide per ml settled beads with tumbling at 4 °C for 30 min. Beads were washed 4 $\times$ and resuspended in buffer A as a 50% suspension. In **a**, 50 μ l glutathione-agarose suspensions were mixed with 80 μ l GTP- γ S-charged cytosolic Ras (lanes 1, 4 and 5), 80 μ l mock GTP-charging reaction (lanes 2, 7), or 20 μ l GTP-charged Ras plus 60 μ l mock GTP-charging reaction (lanes 3, 6). Similarly, 80 μ l GDP- β S-charged Ras (lanes 10, 11, 14), 80 μ l mock GDP-charging reaction (lanes 8, 13), or 20 μ l GDP- β S-charged Ras plus 60 μ l mock GDP-charging reaction (lanes 9, 12) were added to 50 μ l glutathione-agarose bead suspension. Buffer A (0.6 ml) containing (final concentration) 0.2% BSA, 5 mM MgCl₂, 25 μ M ZnCl₂, pH 7.5, was added to all tubes. The suspension was tumbled at 4 °C for 6 h, washed 5 $\times$ in buffer A, eluted into SDS and run on SDS-PAGE, followed by transfer to a PVDF membrane and immunoblotting using pan-Ras monoclonal antibody-2 (Oncogene Science), detected using enhanced chemiluminescence (Amersham). In **b**, membrane-derived and cytosolic Ras preparations were diluted to roughly the same Ras polypeptide concentration, as judged by immunoblot and gel staining, both were charged with GTP- γ S and incubated with immobilized GST fusion proteins as in **a**. A GST-IRS-1 fusion protein was used as control in place of GST. Ras standard polypeptide was from Oncogene Science.

(Fig. 1b). Thus a fully processed Ras polypeptide is not essential for the binding of Ras to the Raf amino-terminal domain.

The intrinsic rate of Ras-catalysed hydrolysis (Fig. 2) of [γ - 32 P]-GTP is not altered by recombinant GST-Raf(1-257). In contrast, the ability of the GTPase-activating protein p120 Ras-GAP to stimulate Ras GTPase can be potentially inhibited by GST-Raf(1-257), whereas GST alone is without effect. The mutant GST-Raf(1-257C168S) at the same concentration gives significantly less inhibition of Ras-GAP than the wild-type GST-Raf(1-257) polypeptide (Fig. 2a). The ability of GST-Raf to inhibit the GAP-stimulated GTPase activity of GTP-Ras was used to gain an estimate of the affinity of GST-Raf(1-257) for GTP-Ras. GST-Raf(1-257) inhibits GAP-stimulated Ras GTPase with an IC_{50} of 0.13 μ M, whereas the IC_{50} for mutant GST-Raf is about double this (Fig. 2c).

Role of the Ras effector domain

Inasmuch as the region on Ras that binds Ras-GAP overlaps the Ras effector domain³⁴, we next evaluated the importance of the Ras effector domain to the interaction of Ras with Raf(1-257). To facilitate our evaluation of Ras variants, we used a yeast expression system designed to detect protein-protein interactions as a result of their ability to reconstitute the transactivating function of the GAL4 protein³⁵. Thus complementary DNA sequences encoding c-Raf(1-257) and the c-Raf(1-257C168S) mutant were inserted into pMA424 (ref. 36), fused in-frame to DNA sequences encoding GAL4 amino-acid residues 1-147, which encompass the GAL4-DNA binding domain.

pACT is a vector that encodes DNA sequences corresponding to the GAL4 transactivation domain II (amino acids 768-881 ref. 37). Co-expression of pMA424 Raf(1-257) or pMA424 Raf(1-257C168S) with pACT in a yeast strain containing a GAL4 binding site (from the upstream activating sequence of GAL1) that drives expression of a *lacZ* reporter gene, did not provide any activation of *lacZ* expression (Fig. 3, panel 1). Fusion of DNA sequences encoding a V12G-Ha-Ras polypeptide in-frame with the GAL4 transactivating domain in pACT led to the appearance of several colonies strongly expressing *lacZ* when cotransfected with wild-type *raf* sequences corresponding to amino acids 1-257, but not mutant *raf* (1-257C168S) sequences. Unequivocal interpretation was prevented, however, by a large decrease in transformation efficiency on expression of the *ras* sequences, whether transfected with wild-type or mutant *raf* N terminus sequence (Fig. 3, panel 2). Inasmuch as a fully processed Ras carboxy terminus did not seem to be critical to the interaction *in vitro* between Raf(1-257) and Ras already described (Fig. 1b), the V12G-Ha-Ras DNA sequences in pACT were modified to remove the four C-terminal amino acids (Δ C). This truncation eliminated the growth inhibitory effect of the pACT-encoded Ras sequences; under these conditions, expression of V12G-Ha-Ras Δ C with wild-type c-Raf(1-257) strongly reconstitutes the GAL4-driven expression of *lacZ*, whereas coexpression of V12G-Ha-Ras Δ C with the mutant Raf(1-257C168S) results in very weak expression of *lacZ* (Fig. 3, panel 3). A similar pattern of *lacZ* expression was observed when the c-Raf(1-257) DNA sequences in pMA424 were exchanged into pACTII and cotransformed with pMA424 containing the Ras or Rap-1b inserts (results not shown).

The preferential binding of Ras to wild-type Raf amino-terminal sequences rather than to mutant Raf (Fig. 3) recapitulates the results of direct binding *in vitro* (Figs 1 and 2). We therefore tested the ability of wild-type Raf(1-257) and mutant Raf(1-257C168S) to interact with a series of V12G-Ha-Ras Δ C structures containing mutations in and around the Ras effector domain, as reflected by reconstitution of GAL4-dependent *lacZ* expression in yeast (Fig. 4; Table 1). The Ras effector domain, generally defined as residues 32-40, was identified as a region essential for transformation, in which mutations do not alter the intrinsic Ras activity of nucleotide binding and hydrolysis, and its membrane localization¹. The integrity of this region is also

TABLE 1 Interference of Ras effector domain mutations with Ras-Raf interaction in yeast in parallel with Ras transformation potency in NIH 3T3 cells

pACTII insert	β -Galactosidase (units $\pm$ s.d.)	Transformation efficiency	Relative NF1/GAP binding
None	0.05 $\pm$ 0.01	—	—
V12-Ha-Ras Δ C	4.43 $\pm$ 0.12	1.0	1.0
V12N38-Ha-Ras Δ C	0.40 $\pm$ 0.01	0.01	0.28/0.87
V12 Δ 34A38-Ha-Ras Δ C	0.11 $\pm$ 0.01	<0.001	<0.001/<0.08
V12E45-Ha-Ras Δ C	ND	<0.001	1.3/3.0
V12G26I27-Ha-Ras Δ C	1.38 $\pm$ 0.03	0.04	3.91/ND
V12E30K31-Ha-Ras Δ C	2.83 $\pm$ 0.02	0.14	0.03/0.16

pMA424raf(1-257) was cotransformed into yeast (as in Figs 3, 4) with the pACTII-encoded V12G-Ha-Ras Δ C variants described for Fig. 4. After 3 days growth on selective medium, plates were scraped and an aliquot of each pool grown in liquid selective medium to an absorbance at 600 nm of 0.8. A whole-cell suspension was permeabilized and assayed in triplicate for β -galactosidase activity using O-nitrophenyl β -D-galactoside⁴⁸. Data on transformation efficiency in NIH 3T3 cells and relative binding to NF1/GAP are taken from ref. 38; transformation data are for full-length V12G-Ha-Ras polypeptides, whereas NF1/GAP binding refers to c-Ras polypeptides. ND, not determined.

crucial for Ras binding to p120 Ras-GAP (ref. 34). Two V12G-Ha-Ras Δ C DNA constructs containing mutations within the effector domain (corresponding to D38N and Δ 34, D38A) known to abrogate Ras binding to GAP as well as Ras transforming activity in NIH 3T3 cells^{34,38} (Table 1), were investigated for interaction with Raf(1-257); both effector mutants failed to activate *lacZ* expression on cotransfection with either the wild-type or mutant *raf* sequences (Fig. 4, G versus B and C; Table 1). Mutation of the Ras residues 26, 27, 30, 31 and 45 adjacent to the Ras effector domain impair Ras transforming activity, despite modest or no impairment in Ras affinity for Ras-GAP (ref. 34). The effects of mutations at these residues, inserted into the V12G-Ha-Ras Δ C DNA sequence, on the activation of *lacZ* expression in collaboration with Raf(1-257) is shown in Fig. 4 (plates A, D and E) and in Table 1. The D45E mutation greatly impairs Ras transforming activity, despite enhanced binding of this mutant Ras to Ras-GAP and NF1, and a normal sensitivity to GAP stimulation of Ras GTPase³⁸. The D45E mutation inserted into the V12G-Ha-Ras Δ C DNA construct markedly reduces the ability of V12G-Ha-Ras Δ C to activate *lacZ* expression in collaboration with c-Raf(1-257) (Fig. 4, compare plates G and A). The alteration of Ras residues N26H27 to G and I, respectively, also spares GAP binding and responsiveness while decreasing Ras transforming activity substantially (although less severely than D45E) (Table 1; refs 34 and 38). Insertion of the G26I27 mutation into the V12G-Ha-Ras Δ C construct diminished the activation of *lacZ* expression on coexpression with Raf(1-257), although to a lesser degree than D45E (Fig. 4, plate D versus A; Table 1). Alteration of Ras residues D30E31 to E and K, respectively, reduces Ras binding to GAP and Ras transforming ability proportionately by 80-90% (Table 1; refs 34, 38). The E30K31 V12G-Ha-Ras Δ C structure exhibits a modestly impaired ability to stimulate *lacZ* expression in collaboration with c-Raf(1-257) (Fig. 4, plate E versus A). Table 1 shows the β -galactosidase activity of the various clones in Fig. 4 and compares this with the transforming and binding activity of the various Ras mutants to p120 Ras-GAP and NF1. From these data it is clear that there is a strong correlation between the ability of these Ras effector domain mutants to transform NIH 3T3 cells and their ability to activate *lacZ* expression by interacting with the c-Raf amino-terminal regulatory domain.

The Rap 1a (ref. 39) and 1b (ref. 40) polypeptides (identical to each other over residues 4-106) are low-molecular-weight GTP-binding proteins identical to Ras in the effector domain (residues 32-40). Rap-1a (Krev-1) was identified as suppressor of the transformed phenotype induced by v-RasK in NIH 3T3 cells³⁷; Rap 1a itself is non-transforming. Rap 1b antagonizes the action of Ras in *Xenopus* oocytes⁴¹. Mutation within the Rap-1 effector domain impairs its ability to act as an inhibitor of *ras*

transformation⁴². It has thus been suggested that the identical effector domain in Rap 1a/1b and Ras may permit binding to a common (Ras) effector, and in fact Rap 1a binds Ras-GAP with high affinity^{43,44}; however, Ras residues 32–40 in the Rap 1a context fail to activate and actually inhibits the Ras signal transduction pathway. In view of these properties, it is noteworthy that V12G-Rap-1b and the V12G-Rap-1b Δ CT interact strongly with Raf(1–257) to activate *lacZ* expression, while showing no interaction with the mutant Raf(1–257C168S) (Fig. 3, panels 4, 5).

Discussion

These findings demonstrate that Raf-1 and Ras can bind to each other directly, as measured *in vitro* using purified polypeptides

and *in situ* using a yeast expression system dependent on physical reconstitution of GAL4 DNA binding and transactivating domains. The c-Raf-1 domain that participates in Ras binding is the c-Raf amino-terminal regulatory region, and the integrity of the c-Raf-1 cysteine finger, a structure crucial for the interaction of Raf with upstream-activating elements, is likewise important for its direct binding with Ras *in vitro* and *in situ*. As regards Ras, an intact effector domain is necessary for interaction with Raf, and mutations in and around the Ras effector domain impair Ras transforming activity in parallel with the loss in ability of Ras to interact with Raf *in situ* (Table 1). The binding of Raf(1–257) to c-Ha-Ras does not alter the kinetics of Ras GTPase, but inhibits the stimulation of Ras GTPase activity by Ras-GAP, consistent with competitive binding of both the Raf-1 and GAP polypeptides to the Ras effector

FIG. 3 Detection of a Ras-Raf(1–257) and Rap-1b-Raf(1–257) interaction in *S. cerevisiae* using the two-hybrid system. The vector pMA424 (ref. 36) containing either Raf(1–257) (left-hand plate in each pair) or Raf(1–257C168S) (right-hand plate in each pair) DNA sequences fused in-frame with sequences encoding GAL4(1–147) was cotransformed into yeast with the pACTII vector containing no insert (panel 1), or DNA sequences encoding V12G-Ha-Ras (panel 2), V12G-Ha-Ras Δ CT (panel 3), murine Rap 1b (panel 4), or Rap1b Δ CT (panel 5) fused in frame with GAL4 transactivation domain 11. After 3 days growth on selective media, colonies were replica-plated to selective media containing 5-bromo-4-chloro-3 indolyl β -D-galactoside (X-gal) and photographed 3 days later.

METHODS. DNA sequences encoding Raf(1–257) and Raf(1–257C168S) were prepared by PCR using plasmids RSV-C4 and RSV-PM17 (ref. 31), respectively, as templates. The 5' terminus was constructed to insert in frame with the GAL4(1–147) sequences at the EcoRI site; the pMA424 *Sal*I site was used for the 3' terminus of the *raf* sequences. The reading frame and the C168S mutation were verified by DNA sequencing. The V12G-Ha-Ras and the Rap1b DNA sequences inserted into pACTII were generated using PCR, and constructed to insert in-frame with the GAL4 transactivation domain 11 sequences using the *Bam*HI and *Xho*I sites. The Δ CT constructs (panels 3 and 5) lacked the DNA sequences encoding the carboxy-terminal four amino acids of Ras and Rap 1b. The Rap 1b DNA sequences were isolated by PCR using a murine T-cell cDNA library. The yeast strain GGY1 : : 171 was transformed with the pMA424 and pACTII vector constructs according to ref. 48, modified by addition of 10% dimethylsulphoxide to the transformation mixture before heat shock. Transformants were grown on SD His⁺Leu⁺ plates as described in refs 35 and 36 for 3 days before replica-plating to X-gal-containing plates, which were photographed after 72 h.

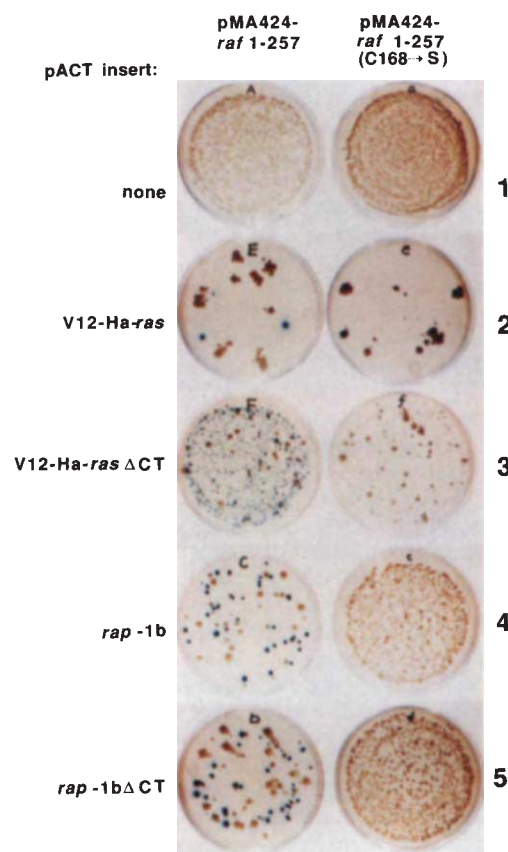
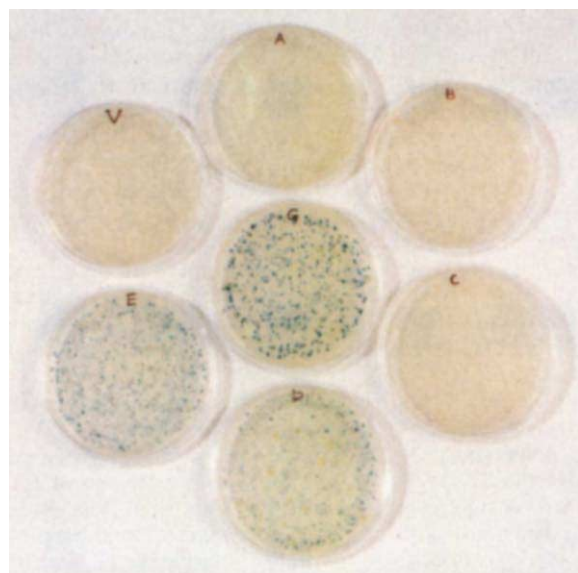


FIG. 4 Mutations in and around the Ras effector domain impair interaction between V12G-Ha-Ras Δ CT and Raf(1–257). The vector pMA424 encoding Raf(1–257) was cotransformed into yeast with the pACTII vector containing either V12G-Ha-ras Δ CT (plate G, centre) or no insert (plate V), or *ras* constructs containing mutations introduced into the V12G-Ha-ras Δ CT background: D45E, plate A; D38N, plate B; delete P34, D38A, plate C; N26H27 to G1, plate D; D30E31 to EK, plate E. After 3 days growth on selective medium, colonies were replica-plated to selective medium containing X-gal and photographed 3 d later. Not shown are parallel cotransformations of the same set of pACTII V12G-Ha-ras Δ CT variants with pMA424raf(1–257C168S); no blue colonies were detected when these transformants were grown on X-gal plates.

METHODS. Methods as for Fig. 3. Variant *ras* sequences were obtained by PCR using the cognate sequence encoded in pMV7 (refs 34, 38) as template. Each pACTII-*ras* construct was verified by DNA sequencing.

domain. The Ras carboxy-terminal domain, including the *S*-farnesyl moiety, does not seem to be crucial for Ras-Raf interaction. The requirement for a fully processed Ras carboxy terminus for the Ras-mediated activation of ERK in *Xenopus* extracts¹² indicates that a role for this domain in Ras activation of Raf-1 kinase is likely to be identified, for example in binding of Ras to the Ras guanyl-nucleotide-exchange protein, or perhaps to other polypeptides involved more directly in Raf activation. Rap 1b also interacts strongly with the Raf amino-terminal domain, providing a plausible mechanism for antagonism between Ras and Rap 1a/b through the generation of a non-productive Rap-1/Raf complex, as occurs with Rap 1a and Ras-GAP (ref. 45).

We have not yet detected activation of c-Raf-1 kinase activity towards MEK on binding of GTP- γ S-loaded Ras to inactive full-length baculoviral c-Raf-1 *in vitro*. Moreover, we have been

unable to detect c-Raf polypeptide in immunoprecipitates of c-Ras from resting or stimulated cells, or vice versa. Given the high affinity of the Ras-Raf interaction that occurs *in vitro*, this negative result may reflect the transient nature of the interaction *in situ* or a loss of the interaction after cell disruption. Nevertheless, in view of the evidence identifying c-Raf-1 as an indispensable downstream effector of the mitogenic response to Ras, our results strongly indicate that the c-Raf-1 kinase is a direct target of the active Ras polypeptide; the Ras-Raf interaction described here is probably the first step in the Ras-mediated activation of c-Raf-1 kinase *in situ*.

Finally, the importance of the c-Raf cysteine finger in the Raf-Ras interaction suggests that homologous domains in other non-nuclear signal transduction proteins such as protein kinase C, n-chimaerin or diacylglycerol kinase, may also enable these proteins to interact directly with Ras or other Ras-like polypeptides. $\square$

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LETTERS TO NATURE

Inner spiral structure of the galaxy M51

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MODELLING the structure and kinematics of spiral galaxies requires accurate maps of the mass-tracing stellar population. But this has hitherto been difficult because of dust obscuration and the presence of luminous young stars. To minimize the effects of dust and maximize sensitivity to the dominant stellar population, we have obtained K-band (2.2- μ m) images of the nearby 'grand-design' spiral galaxy NGC5194 (M51). Our observations reveal remarkable dynamical structures not visible in conventional optical images. We find that the spiral arms extend significantly further towards the galaxy's centre than previously observed and can be traced continuously through almost three revolutions – roughly twice as far as with optical images. The coherence of the arms over this large radial range challenges current theories of spiral structure. We suggest that a combination of several mechanisms, such as the interaction of M51 with the neighbouring galaxy NGC5195, forcing by the

central 'bar', or distortions from density waves, is required to generate the observed structure.

One of the most striking characteristics of many disk galaxies is their spiral pattern. This pattern is often roughly a two-arm structure, but can be more complex. Even among the grand-design spirals, it is difficult to follow unambiguously the winding of any arm through more than one full revolution. In photographs of one of the nearest grand-design spiral galaxies, NGC5194 (M51), the arms can be traced through about 540°. M51 is a popular test case for models of spiral structure as it is relatively nearby^{1,2} (8 Mpc) and nearly face-on³ (inclination of 20°), and has a large amplitude two-arm structure⁴. Of the several mechanisms proposed to explain how such spiral patterns are created and maintained⁵, the density wave theory^{6,7} has the most support because of its generality and correspondence with the observed structure and kinematics of spiral arms (for an analysis of the arm structure in M51, see ref. 3). But it is almost certain, at least for M51, that some other mechanism such as tidal torquing or bar forcing is partially responsible for the arms. Most unbarred differentially rotating grand-design spirals, including M51, do in fact have a nearby companion galaxy⁸.

The mapping of the stellar population which contributes most of the mass in the disks of spiral galaxies is distorted by the obscuring effects of dust and by the presence of luminous young stars. To minimize the effects of dust and maximize our sensitivity to the dominant stellar population in the disk, we obtained images at 2.2 μ m of the central region of M51. The data

were obtained with the Steward Observatory 2.3-m telescope using a charge-coupled device (CCD) for B and I images (at 0.45 and 0.8 μ m respectively) and a Nicmos 3 (256 $\times$ 256) array for J and K images (2.2 μ m). Exposure times range from 2 min (CCD) to 45 min (Nicmos 3) and the data were reduced using standard techniques. The most prominent features visible in the K-band image (Fig 1) are the spiral arms, extending inward to about $r = 30''$, as in the optical images, and an oval central component which had been identified in earlier optical work^{9,10}. Closer examination reveals the presence of spiral structure well inside 30''.

To investigate the inner spiral structure, we removed the overall luminosity gradient by fitting a smooth model to the oval component in the K-band image and subtracting it from all images. Before subtraction, this model was normalized to produce zero mean residual in each image. The model's mean position angle differs by 35° from the position angle of the disk of the galaxy. This position angle offset indicates that the apparent ellipticity (a maximum of 0.22 at 10'') is not a direct result of projection. The residual images for the three passbands are shown in Fig 2. Distortions created by dust and young star clusters render the B and I images useless for tracing the inner arm structure. We examined J–K colour images and determined that the new features seen at K are not caused by dust extinction but truly represent ridges in the stellar mass distribution. The K-band residual image shows that the spiral arms wind through an additional 540° beyond that seen in the B-band images of the entire galaxy, and end at an angular distance of about 10'' from the centre of the galaxy.

The innermost region ($r < 10''$) appears to be either a small bar or an oval bulge. The measured ratio of the minor to major axis, b/a , of the central component is 0.5, which is significantly less than that expected for an axisymmetric structure seen in projection ($b/a = 0.94$). We believe that this component is most likely to be a bar and not an oval bulge, because the projected brightness profile (Fig. 3) shows a well-defined break or shoulder at the radius where the central component terminates (about 10'').

Some constraints on analytical models of spiral structure are derived from the location and strength of dynamical resonances. Resonances occur at radii where the period of the noncircular component of a star's orbit is an integral multiple, m , of the tumbling period of the non-axisymmetric gravitational potential of the galaxy. Although the study of disk dynamics is complex, the phase coherence between the gravitational force and the star's position is a key ingredient in disk dynamics and most models for density waves predict that the spiral arms extend from the inner to the outer Lindblad resonance ($m = 1, -1$ respectively, where $m < 0$ indicates that the noncircular motion is in the opposite sense to the circular motion). In the data

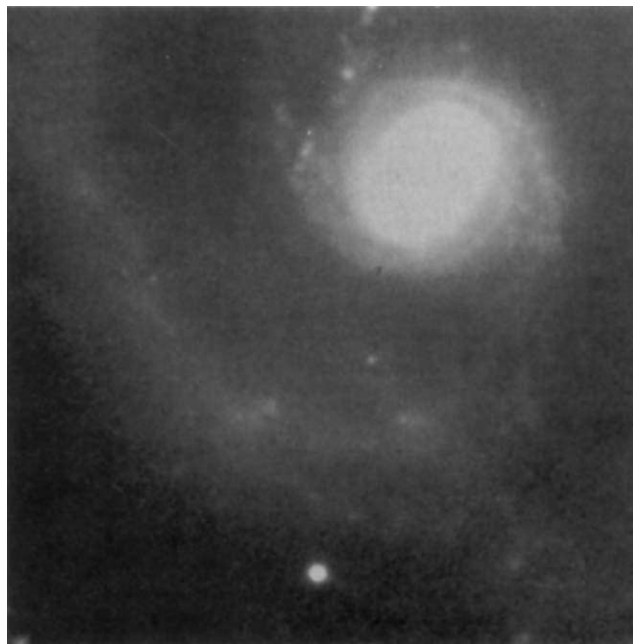


FIG. 1 K-band image of the central portion of M51. The field of view is almost 145'' across (5.6 kpc).

presented here, the spiral arms continue well inside the estimated position of the inner Lindblad resonance (1.6 kpc; ref. 3), although there is a minimum in the arm amplitude near this position⁴. However, because the pattern speed is not directly observed and because the rotation curve in the inner region is poorly defined, the position of the inner Lindblad resonance is uncertain. The uncertainties in the inner rotation curve also make a study of differential rotation in the inner region highly uncertain.

Computer simulations provide an alternative test, but it has been difficult to reproduce the extent and phases of spiral arms evident in the optical images (see ref. 11 for a review). The additional information from the new K-band images compounds these difficulties. Both the optical appearance of the M51/NGC5194 galaxy pair and the extended tail of H I associated with M51¹² imply that a strong interaction is occurring. Although the first numerical simulations of the interaction^{13,14} were unable to account for the spiral structure between 30'' and 60'', the inclusion of the disk's self gravity in the subsequent simulations greatly improved the qualitative agreement between the simulations and observations¹⁵. The arms in the inner disk (30''–60'') seem to be a result of swing-amplification of weak kinematic waves in the outer part of the disk¹⁶, possibly in combination

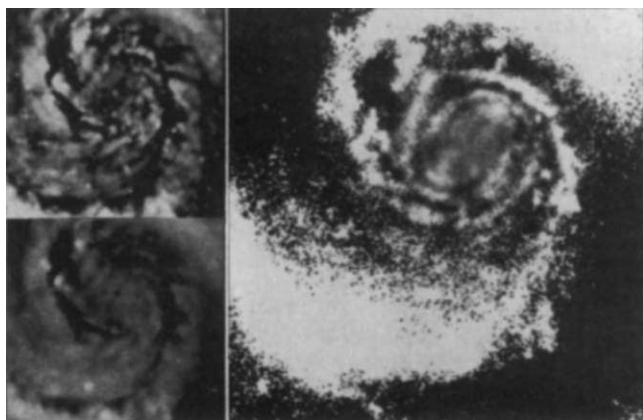


FIG. 2 B (upper left), I (lower left), and K (right) residual images. Notice that the inner spiral pattern is only visible in the K-band image. The field of view in the K-band image is 115'' (4.5 kpc). The arms begin at a radius of about 400 pc.

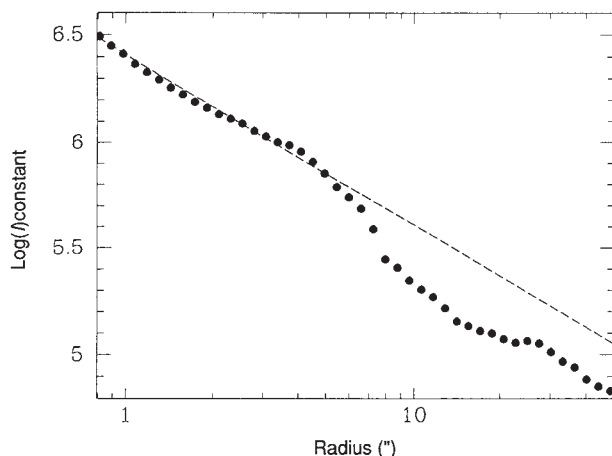


FIG. 3 Radial brightness profile along isophotes as a function of semi-major axis length of isophote. The dashed line represents an $r^{-1/4}$ brightness profile fit to the inner region.

with existing spiral structure. The advantage of considering such an interaction is that it is a transient phenomenon and so one might expect fewer difficulties in accounting for spiral structure inside the position of the inner Lindblad resonance. However, no simulation has yet produced or predicted the spiral structure observed in our K-band images.

Simulations aimed at examining the innermost spiral pattern will require higher spatial resolution than previously obtained and will be extremely sensitive to the model of the inner disk (depending, for example, on the assumed rotation curve and gas fraction). Can the interaction between the two galaxies generate the inner spiral arms and the mini-bar? If so, then this shows that even moderate interactions (a mass ratio of 1:2, but a pericentre passage of 5 to 6 exponential scale lengths¹¹) can affect the primary galaxy's structure well into the centre.

The data presented here show that the spiral arms in M51 wind coherently through about three revolutions. The dynamics of the M51/NGC5194 system are complex, although the outer regions are clearly shaped by tidal effects from the interaction with NGC5195. Whether this tidal interaction can generate a spiral wave that propagates almost to the centre, and whether it can also account for the mini-bar, are questions that remain unanswered until more detailed simulations are completed. Studies of other galaxies using infrared imaging may uncover similar fine structure in the inner, and previously unexplored, portion of their disks. □

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A new family of copper oxide superconductors $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ stabilized at high pressure

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THE search for new copper oxide superconductors¹ has been pursued mainly by exploring a range of chemical compositions (counter-cations and oxygen content) and reaction temperatures. We have explored the effects of an additional variable, the reaction pressure, and have thereby synthesized some new compounds, including $(\text{Ca}_{1-y}\text{Sr}_y)_{1-x}\text{CuO}_2$ which shows superconductivity at 110 K (refs 2, 3). Here we report the synthesis of a new family of high-pressure phases with the formula $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ ($n=1, 2, 3, \dots$) extending from $\text{Sr}_2\text{CuO}_{3.1}$ ($n=1$) to SrCuO_2 ($n=\infty$)⁴. $\text{Sr}_2\text{CuO}_{3.1}$ and $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$ ($n=2$) are superconductors with transition temperatures (T_c) of 70 and 100 K, respectively. The metal sublattice of $\text{Sr}_2\text{CuO}_{3.1}$ is of the tetragonal K_2NiF_4 (or Nd_2CuO_4) type with lattice constants $a=3.764 \text{ \AA}$ and $c=12.548 \text{ \AA}$, and iodometric measurements suggest that nearly half of the oxygen atoms are missing from the counter-layers to $\text{Sr}_2\text{O}_{1.1}$. Homogeneous pyramidal or octahedral coordination, which has generally been believed to be indispensable to attain T_c s of $\sim 100 \text{ K}$, is thus absent. The $n=2$ and 3 members seem to correspond to the high-pressure phases in the Ca–Sr–Cu–O system recently reported by Adachi *et al.*⁵.

Sr_2CuO_3 , SrCuO_2 and $\text{Sr}_{14}\text{Cu}_{24}\text{O}_{41}$, the phases stable in the Sr–Cu–O system at ambient pressure⁶, all lack the two-dimensional CuO_2 sheets thought to be a key structural component for high-transition-temperature (high- T_c) superconductivity. In the case of SrCuO_2 , however, pressure induces a structural transition to the denser infinite-layer structure in which CuO_2 sheets sandwich single Sr layers⁴. Modification of the chemical formula to $\text{Sr}_{1-x}\text{CuO}_2$ makes it superconduct at $\sim 100 \text{ K}$ (ref. 7). Orthorhombic Sr_2CuO_3 containing Cu–O chains can be oxidized to tetragonal $\text{Sr}_2\text{CuO}_{3.9}$ at 16 MPa of oxygen⁸, whereas the oxygen-deficient $\text{CuO}_{1.9}$ sheets in this compound did not show either superconductivity or metallic conductivity. More recent results indicated that multi-phase samples prepared at 5 GPa from a starting composition of $\text{Ca}_{0.33}\text{Sr}_{0.6}\text{CuO}_{2+\delta}$ became superconducting at $T_c \sim 90\text{--}100 \text{ K}$ (ref. 5). Adachi *et al.* suggested that new phases with crystallographic periodicities of 10.3 Å and 13.6 Å were responsible for the superconductivity.

The starting materials for our high-pressure synthesis were Sr_2CuO_3 and finely powdered CuO. Appropriate proportions of these were placed in gold capsules (20 mm³ volume) and pressed almost isostatically up to 6 GPa using a cubic anvil apparatus³. The temperature was ramped to $\sim 1073\text{--}1173 \text{ K}$ in 5 minutes, held constant for 30 minutes, and finally dropped to $\sim 400 \text{ K}$ in a few seconds and to room temperature in a minute. To provide an oxidizing atmosphere KClO_4 , which releases oxygen on heating, was added to the capsule or mixed with the starting powder.

In the absence of KClO_4 , orthorhombic Sr_2CuO_3 remained stable under pressure. In an oxidizing atmosphere, in contrast, it was transformed into superconducting tetragonal $\text{Sr}_2\text{CuO}_{3+\delta}$. The sample is almost single phase, as can be seen from powder X-ray diffraction (Fig. 1a). All the intense peaks can be indexed assuming a tetragonal cell with $a=3.764 \text{ \AA}$ and $c=12.548 \text{ \AA}$. The accompanying peaks marked with asterisks indicate a superlattice of $4\sqrt{2}a \times 4\sqrt{2}a \times c$. This is supported by electron diffrac-

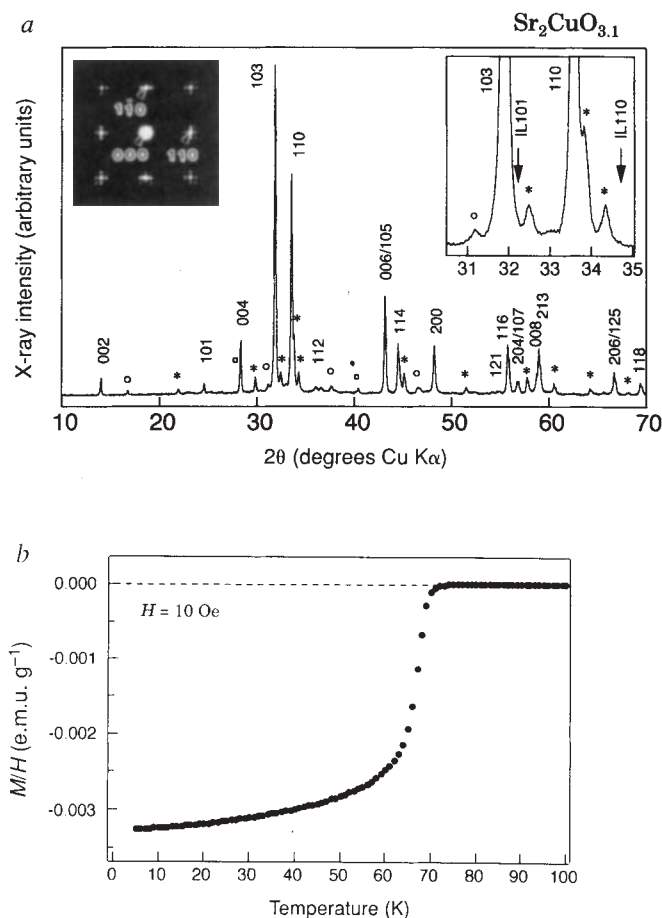


FIG. 1 *a*, X-ray and electron diffraction patterns for $\text{Sr}_2\text{CuO}_{3.1}$. The indexed peaks and spots are the fundamental reflections of the K_2NiF_4 -type structure, and the accompanying ones (those marked with asterisks in the XRD pattern) come from a superlattice of $4\sqrt{2}a \times 4\sqrt{2}a \times c$. Small amounts of $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$ (circles) and KCl (squares) are mixed in the XRD pattern. The right-hand inset is an enlarged XRD pattern showing the absence of the infinite-layer phase—the positions where the two main peaks should appear are indicated by arrows. *b*, Temperature dependence of magnetic susceptibility (M/H) measured for a powdered sample of $\text{Sr}_2\text{CuO}_{3.1}$ in an applied field of 10 Oe on cooling.

tion measurements with various incident beam axes. A typical diffraction pattern taken at $[001]^*$ incidence is shown in the inset to Fig. 1*a*, where the intense fundamental peaks have smaller satellite spots. The sample contained small amounts of $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$, the $n=2$ member of the series, with a tetragonal unit cell of $a=3.902$ Å and $c=21.085$ Å. It also contained KCl. The absence of infinite-layer SrCuO_2 can be seen from the right-hand inset to Fig. 1*a*, where arrows indicate the positions of the two main peaks of this phase. Moreover, no intergrowth of the infinite-layer type structure was observed in the electron microscope. Figure 1*b* shows magnetic susceptibility (M/H , where M is magnetization and H is magnetic field) of a powdered sample, indicating a sharp superconducting transition at $T_c \sim 70$ K. The superconducting volume fraction estimated at 5 K is about 22%, which is large enough to constitute bulk superconductivity.

The X-ray and electron microscope results show that the metal sublattice is of the K_2NiF_4 type or the Nd_2CuO_4 type. Preliminary Rietveld analysis of the X-ray diffraction pattern indicates that the K_2NiF_4 -type arrangement is more probable for the oxygen sublattice, but detailed structural analysis by means of high-resolution neutron diffraction would be necessary to conclude this unambiguously. The oxygen content determined by iodometric titration is only 3.1. This means that nearly half the oxygen atoms are missing from the ideal Sr_2O_2 layers, if the formation of two-dimensional CuO_2 sheets is assumed from the

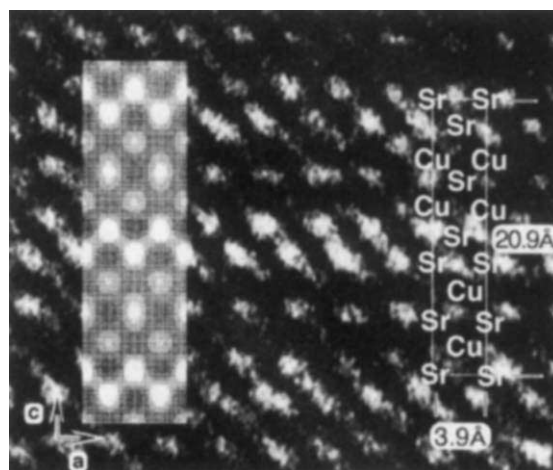


FIG. 2 High-resolution electron microscopy image of $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$ ($n=2$). The simulated image inserted was calculated by assuming the $\text{La}_2\text{SrCu}_2\text{O}_6$ type structure with 40% oxygen vacancies in the NaCl-type Sr_2O_2 layers, for a crystal thickness of 37.65 Å and a defocus value of 525 Å.

superconducting behaviour. The superlattice formation is probably due to ordering of the oxygen vacancies in the counter-layers.

Other members of the $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ family have not yet been isolated. The $n=2$ and 3 members have been identified as macroscopic phases by X-ray diffraction measurements, and phases with $n \geq 4$ have been observed in the electron microscope. $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$ crystallizes in the tetragonal form ($I4/mmm$) with $a=3.9$ Å and $c=21.0$ Å, whereas $\text{Sr}_4\text{Cu}_3\text{O}_{7+\delta}$, seems to have a quasi-tetragonal cell with $a=3.9$ Å and $c=27.8$ Å. These lattice constants vary slightly with excess oxygen content. A high-resolution electron microscope image of $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$ is shown in Fig. 2 (operating voltage 200 kV; JEOL-2000EX). The incident electron beam was parallel to the a axis, giving a cross-sectional atomic view of the layered structure. Metal sites appear as dark dots, oxygen sites as bright dots. From a computer image simulation using the multi-slice method, we identified Sr and Cu atoms as indicated in the micrograph. The metal sublattice consists of Sr double layers alternating with the Cu/Sr/Cu slabs along the c axis, and is thus isomorphous with those of $\text{La}_2\text{SrCu}_2\text{O}_6$ and $\text{Sr}_3\text{Ti}_2\text{O}_7$ (ref. 9).

The temperature dependence of magnetic susceptibility obtained from a sample mainly containing the $n=1$ and $n=2$

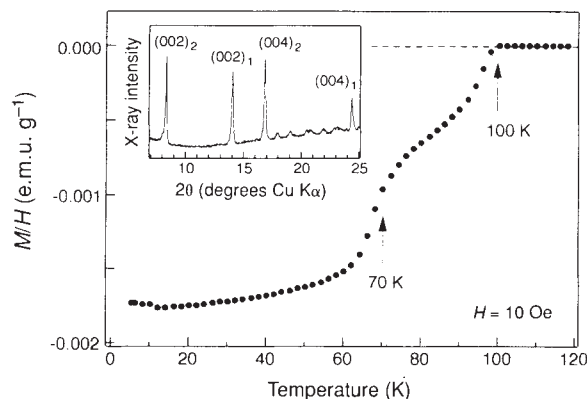


FIG. 3 Temperature dependence of magnetic susceptibility (M/H) of a sample mainly containing $\text{Sr}_2\text{CuO}_{3.1}$ and $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$. Shown in the inset is a low-angle part of the XRD pattern.

members shows a two-step transition at 70 K and 100 K (Fig. 3). The low-angle X-ray diffraction pattern of this sample has two series of reflection peaks $(00l)_n$ (Fig. 3, inset). No trace of the infinite-layer compound was detected by X-ray diffraction. The transition temperature of the $n=2$ member is thus estimated to be 100 K.

The superconducting transition of $\text{Sr}_2\text{CuO}_{3.1}$, observed from resistivity measurements using the four-terminal method, was much less sharp than expected from the magnetic transition, as shown in Fig. 4 (upper curve). We attribute this to precipitation of impurities at the grain boundaries. Most of the impurities would be SrO_2 , as detected by electron microscopy. Partial substitution of Ca for Sr improved the resistivity behaviour (Fig. 4, lower curve, measured for a sample mainly containing $(\text{Ca}, \text{Sr})_2\text{CuO}_{3+\delta}$ and $(\text{Ca}, \text{Sr})_3\text{Cu}_2\text{O}_{5+\delta}$ with a Ca:Sr ratio of 0.3:0.7).

The stacking sequence of the phases is $\text{Sr}_2\text{O}_{1+\delta}/\text{Cu}^{(1)}\text{O}_2/\text{Sr}/\text{Cu}^{(2)}\text{O}_2/\dots/\text{Cu}^{(n-1)}\text{O}_2/\text{Sr}/\text{Cu}^{(n)}\text{O}_2/\text{Sr}_2\text{O}_{1+\delta}$, if the existence of CuO_2 sheets is assumed. For example, the first member $\text{Sr}_2\text{CuO}_{3+\delta}$ has the $\text{Sr}_2\text{O}_{1+\delta}/\text{CuO}_2/\text{Sr}_2\text{O}_{1+\delta}$ sequence. The pressure-induced transformation in an oxidizing atmosphere from orthorhombic Sr_2CuO_3 ($\text{Sr}_2\text{O}_2/\text{CuO}/\text{Sr}_2\text{O}_2$ sequence) into tetragonal $\text{Sr}_2\text{CuO}_{3+\delta}$ implies that the CuO_2 sheets contract on oxidation, making the specific density of $\text{Sr}_2\text{CuO}_{3+\delta}$ higher than it would be if the oxygen-filled Sr_2O_2 layers were preserved as $\text{Sr}_2\text{O}_2/\text{CuO}_{1+\delta}/\text{Sr}_2\text{O}_2$. Our results suggest that two-dimensional CuO_2 sheets are stabilized under high pressure, as noted in previous studies¹⁰.

The Ruddlesden-Popper series is well known in crystal chemistry. The general formula is $\text{A}_{n+1}\text{M}_n\text{O}_{3n+1}$ where A represents Ca, Sr, Eu and M represents Mn, Ti. The endmembers crystallize in the K_2NiF_4 ($n=1$) and perovskite structures ($n=\infty$)¹¹. The present series of cuprates can be considered as an oxygen-deficient version of this series with endmembers $\text{Sr}_2\text{CuO}_{3+\delta}$ ($n=1$) and infinite-layer SrCuO_2 ($n=\infty$). The compounds reported by Adachi *et al.*⁵ with periodicities of 10.3 and 13.6 Å are likely to be the Ca-substituted $n=2$ and 3 members, respectively. In the light of the present study, these periodicities should be reinterpreted as (002) reflections, arising from the planes at $z=c/2$ in the body-centred tetragonal lattice. According to our experiments, the $n=1$ member was eliminated by Ca-substitution.

We have not yet obtained transport data clarifying the carrier sign, but the fact that $\text{Sr}_2\text{CuO}_{3.1}$ and others form only in the oxidizing atmosphere is a strong indication that the present compounds are p -type superconductors. The $\text{Sr}_2\text{O}_{1+\delta}$ layer acts as a charge-controlling unit injecting hole carriers into the CuO_2 sheets. The nominal copper valence determined in $\text{Sr}_2\text{CuO}_{3.1}$ by iodometric titration is 2.2, near the optimum carrier concentration of ~ 0.16 per Cu atom to obtain the maximum T_c (ref. 12).

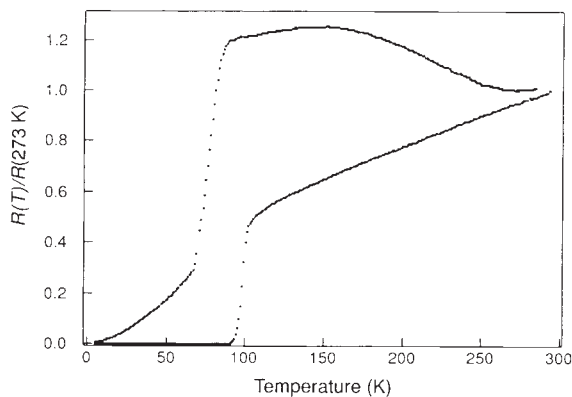


FIG. 4 Temperature dependence of resistivity ($R(T)$) normalized at 273 K measured for an almost monophasic sample of $\text{Sr}_2\text{CuO}_{3.1}$ (upper curve) and a 30% Ca-substituted multiphase sample containing the $n=1$ and $n=2$ members.

The possibility that potassium originating in KClO_4 might substitute partially for strontium is not completely ruled out, but nearly the same bulk superconductivity was observed when SrO_2 was used instead of KClO_4 as an oxygen source.

The most interesting result from the viewpoint of the relationship between structure and high- T_c superconductivity concerns the copper coordination. As the CuO_2 sheets are sandwiched by $\text{Sr}_2\text{O}_{1+\delta}$ layers in $\text{Sr}_2\text{CuO}_{3+\delta}$, nearly half of the apical oxygen atoms are missing in comparison with the case of $(\text{La}, \text{Sr})_2\text{CuO}_4$. For $\text{Sr}_3\text{Cu}_2\text{O}_{5+\delta}$, there should be a considerable proportion of four-fold-coordinated Cu ions mixed with the pyramidally coordinated ones. Inhomogeneous coordination of this kind has not previously been known in p -type superconductors. If the oxygen distribution in the $\text{Sr}_2\text{O}_{1+\delta}$ layers were basically of the fluorite type (as in Nd_2CuO_4) there would be no apical oxygen atoms. We need not assume homogeneous apical oxygen coordination to raise T_c to 100 K. This is consistent with our recent results³ on the infinite-layer superconductor $(\text{Ca}_{1-x}\text{Sr}_x)_{1-x}\text{CuO}_2$, which we consider to be a 110 K p -type superconductor with non-oxygen-capped CuO_2 sheets only. □

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Effect of electrolytes on bubble coalescence

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THE foaminess of ocean waves, relative to fresh water, has long been attributed to the effect of salts in reducing bubble coalescence¹. This phenomenon is exploited in extraction processes using froth flotation², in which the extraction efficiency increases as the bubble size gets smaller. But whereas the bubble-stabilizing effect of surfactants is well understood, the effect of salts is not; the fact that salts decrease the surface tension of water and that they are desorbed from the air–water interface would, if anything, be expected to destabilize bubbles. Here we report the results of experiments conducted to study the stabilization of bubbles by salts. We find that bubble coalescence is inhibited by some salts whereas others have no effect and that this inhibition occurs only upon the ‘matching’ of a two-valued empirical property assigned to each anion and cation. We believe these observations can be explained only by the local influence of the ions on water structure, possibly in a way related to the hydrophobic interaction^{3–8}.

Our apparatus consists of a vertical glass column containing a high density of rising nitrogen bubbles produced at a sinter at the base of the column, and a collimated light source. Bubble

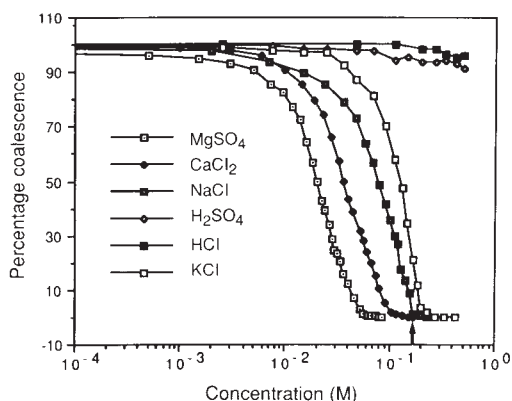


FIG. 1 The percentage coalescence observed for a stream of nitrogen gas bubbles rising in a range of aqueous electrolyte solutions at 21–23 °C. 100% coalescence corresponds to no change in opacity relative to pure water; 0% coalescence is at the point where no further change in opacity was observed.

coalescence was monitored by a photodiode that detects the amount of light transmitted through the column at a set height above the frit. The water was purified by passing it through an activated charcoal column and a reverse osmosis membrane before a single-stage distillation, then stored in a laminar flow filtered air cabinet. Salts were of analytical grade and where possible were roasted before use. The acids were of analytical grade and were used as purchased. The possibility of surfactant contamination can be excluded because the column acts as a flotation cleaning cell. The system gave reproducible results on addition of electrolytes from a burette at the top of the column. This either produced a sharp transition with reduction in bubble sizes, giving a substantially more opaque column; otherwise the electrolyte gave no effect whatever. Multiple bubble collisions during passage through the column appear to increase the sensitivity of the transition, compared with the single-collision studies reported earlier⁹.

Typical results are shown in Fig. 1. The relative light intensity values have been converted to percentage coalescence, where 100% corresponds to no change relative to water and 0% to the point where no further change in opacity was observed. Light intensity was measured ~5 cm above the frit, following the passage of bubbles through a turbulent region which promoted coalescence. It was observed that below this region the addition of salt had no observable effect. This indicates that the effect of salt was not due to a change at the surface of the hydrophilic glass frit. As reported earlier, multivalent electrolytes reduce coalescence at a lower concentration than univalents². All scale with the corresponding ionic strength (or Debye length) rather than concentration.

What is new, however, is that some electrolytes and some mineral acids have no effect at all on bubble coalescence. Figure 2 lists a series of anions and cations that brings the effects of different salts to order. There is a remarkable correlation evident between the ion pairing and their effect. A property (α or β) can be assigned to each anion or cation. The combination ($\alpha\alpha$) or ($\beta\beta$) inhibits coalescence, ($\alpha\beta$) or ($\beta\alpha$) has no effect. Thus NaCl ($\alpha\alpha$) and CH₃COOH (acetic acid) ($\beta\beta$) inhibit coalescence, but CH₃COONa ($\alpha\beta$) and HCl ($\beta\alpha$) do not. No exceptions have yet appeared. In any event, the phenomenon is evidently not explicable in terms of any theory based on the primitive model of electrolytes, and must involve water structure. That conclusion is reinforced by studies we have carried out on sugars; glucose and fructose have no effect, whereas sucrose does.

Earlier work⁹ focused on electrolytes that show coalescence inhibition. We have seen that some acids and salts have no effect whatever. This last admits no obvious explanation. Previous attempts to explain the limited range of results then extant involved increased solution viscosity that would increase hydrodynamic repulsive forces. That argument cannot be sustained: KCl reduces solution viscosity, H₂SO₄ increases it.

In the collision process between bubbles it is clear that coalescence lowers the total free energy by reducing the area of the air–water interface. Opposing this thermodynamic force that favours coalescence, there must arise for dynamic bubble collisions a substantial hydrodynamic repulsion. This is due to the energy required for drainage of the intervening water film and will be significant at separations of typically less than about 10 nm. There must exist a sufficiently strong attractive force between air bubbles in water to account for the observed coalescence. An electrostatic repulsion due to selective ion adsorption at the air–water interface is unimportant¹⁰ over the electrolyte levels studied, where Debye lengths are typically about 1 nm. At small separations of the order of 5–10 nm, attractive van der Waals forces will operate to overcome the repulsive forces. But this force is hardly affected by the levels of salt used and the hydrodynamic repulsion is substantial at separations >10 nm¹¹.

The only remaining attractive force operating between bubble surfaces is the long-range hydrophobic interaction^{2, 8}. It has been measured, can have a range of up to 150 nm between solid hydrophobic surfaces in water, and is 10 to 100 times larger in magnitude than any van der Waals force. It has also been observed between a hydrophobic solid and an air bubble¹². The origin of this force is not understood, but its existence and reproducibility is not in doubt.

How should electrolytes affect the hydrophobic interaction? The limited data available show that the long-range hydrophobic interaction between solid surfaces is reduced by the addition of simple salts like KBr^{4,6,8}. For bubbles also, salts reduce the range of the attractive force. At electrolyte concentrations above our

		Cations								
		β	α	α	α	α	α	β	α	
Anions		H ⁺	Mg ²⁺	Na ⁺	Ca ²⁺	K ⁺	NH ₄ ⁺	Cs ⁺	Me ₄ N ⁺	Li ⁺
α	OH ⁻	✗	I. Sol		I. Sol	✓				
α	Cl ⁻	✗	✓	✓	✓	✓	✓		✗	✓
α	Br ⁻	✗		✓		✓		✓		
α	NO ₃ ⁻	✗		✓	✓	✓	✓		Unavail	✓
β	ClO ₃ ⁻	Unavail	Unavail	✗	Unavail	I. Sol	Unavail	I. Sol	Unavail	Unavail
α	SO ₄ ²⁻	✗	✓	✓	I. Sol		✓			✓
β	ClO ₄ ⁻	✓	✗	✗	Unavail	I. Sol	✗	I. Sol	Unavail	Unavail
β	CH ₃ COO ⁻	✓	✗	✗		✗	✗	✗	✓	
α	Oxalate ²⁻	✗	I. Sol	I. Sol	I. Sol	✓	I. Sol	Unavail	Unavail	

Combining Rules: $\alpha\alpha$ or $\beta\beta$ gives ✓ $\alpha\beta$ or $\beta\alpha$ gives ✗

I. Sol=Insufficiently soluble

Unavail=Salt unavailable

Addition of salt: Prevents coalescence ✓ Has no effect on coalescence ✗

FIG. 2 Effect of electrolytes on bubble coalescence.

transition point, the effect on bubble interactions is to reduce the range of the attractive force from ~ 100 nm to 55 nm¹³. It seems that such salts act to prevent bubble coalescence by reducing the range of the attraction between bubbles. Any explanation for the combining rules that emerge from the table shown in Fig. 2 must then be directly related to the effect of electrolytes on the hydrophobic interaction. As such, this table may provide insight into the mechanism of the hydrophobic interaction.

Finally, it is interesting to note that the salt level in the human body, marked by an arrow in Fig. 1, corresponds to the concentration where the onset of maximum coalescence prevention was achieved in the bubble chamber experiments. This surprising result indicates a possible link between salt levels and susceptibility to decompression sickness, as small bubbles present in tissue do less damage than larger bubbles. □

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Reconstructing sea surface temperature and salinity using $\delta^{18}\text{O}$ and alkenone records

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THE oxygen isotope ($\delta^{18}\text{O}$) composition of foraminiferal tests from deep-sea sediments is widely used as a palaeoclimate proxy, but it includes contributions from sea surface temperature, global ice volume and local salinity, which are difficult to separate. Recently a new technique for deriving palaeotemperatures has been developed which is based on the abundance ratios of unsaturated alkenones in phytoplankton algae^{1,2}. Here we use a combination of oxygen isotope and alkenone records in a deep-sea core from the juncture of the Arabian Sea and the Bay of Bengal to extract the salinity signal from the former record. Variations in salinity are related to the balance between evaporation and precipitation³, and are thus a sensitive indicator of climate change. Our 170-kyr salinity record enables us to reconstruct changes in the Indian monsoon over this period, considerably extending earlier studies (which reached back to 18 kyr ago)^{4–8}. Like these previous studies, we find that large variations in the monsoon occurred during the transition from the last glacial period to the present interglacial, but our results also provide a view of the monsoon throughout the last glacial and

demonstrate the potential of this approach for reconstructing palaeosalinity.

Variations of sea surface salinity are strongly related to the evaporation–precipitation ($E-P$) balance³ and thus represent a sensitive climate indicator. Due to extreme monsoonal atmospheric and oceanographic gradients^{9,10} (Fig. 1), the Northern Indian Ocean is characterized by two main surface water masses: high-salinity surface water (35–36.5‰) due to strong evaporation in the Arabian Sea and low-salinity water (31–34‰) in the Bay of Bengal caused by high precipitation and large river runoff, particularly during the SW monsoon¹¹.

In order to reconstruct climate variability in the Equatorial Indian Ocean, Core MD900963 (05° 04' N, 73° 53' E; 2,450 m water depth; Fig. 1) was recovered on the eastern shoulder of the Maldives Islands (SEYMAMA expedition, 1990; RV *Marion Dufresne*). Situated between the Arabian Sea and the Bay of Bengal, Core MD900963 is probably ideally located to monitor the changes of the monsoon during the last glacial cycle. A detailed $\delta^{18}\text{O}$ stratigraphy was established on the surface dwelling planktonic foraminifer *Globigerinoides ruber* (white) for the 54-m long piston core (Fig. 2b). The timescale was obtained¹² by tuning the $\delta^{18}\text{O}$ record to the SPECMAP record¹³.

We measured the alkenone unsaturation ratio $U_{37}^{K'}$ (refs 1, 2) on the uppermost 10 m of the core to determine sea surface temperature (SST) variations for the last 170,000 years using the temperature equation of Prahli *et al.*¹⁴. Alkenone measurements were carried out at intervals of 10 cm at the Department of Geoscience of the University of Bremen. Sample preparation and technical details are described elsewhere¹⁵. Preliminary observations of the coccoliths indicate that *Emiliania huxleyi* and other species of the family *Gephyrocapsae* are abundant in the first 10 m of Core MD900963 (L. Beaufort, personal communication).

SST variations during the last 170,000 years are rather small—between 25.5 and 28 °C (Fig. 2a). Low SST values of ~ 25.5 – 26.5 °C are observed in oxygen isotope stages 4 and 3 with a minimum centred within stage 3. Interglacial stage 5 is characterized by high SST's between 27 and 28 °C. For stage 6, the SST's are ~ 1.5 °C higher than those observed in the stage 3 minimum.

Our data agree with SST reconstructions based on foraminifera assemblages from the tropical Indian Ocean¹⁶ and in particular with the record published by Clemens *et al.*¹⁷. The fact that these two SST time series (both obtained in the tropical

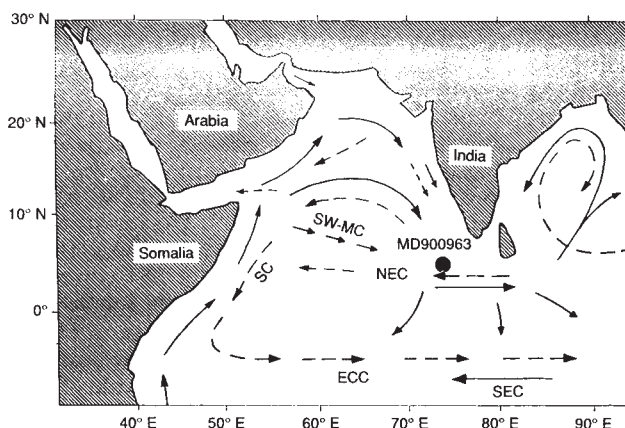


FIG. 1 Circulation patterns in the northern Indian Ocean¹⁰ and location of Core MD900963. The solid lines represent the oceanic circulation during the SW monsoon (May to October), stippled lines the circulation during the NE monsoon (November to April). SC, Somali Current; NEC, North Equatorial Current; ECC, Equatorial Counter Current; SW-MC, SW Monsoon Current; SEC, South Equatorial Current.

Indian Ocean) are similar gives more confidence in the reliability of the U_K method. In particular, both records suggest that stage 6 was relatively warm and definitely not as cold as stage 3.

Changes in $\delta^{18}O$ recorded in planktonic foraminifera are controlled by global variations of seawater ($\delta^{18}O_{WG}$) due to the growth of continental ice sheets, and by local SST and $E-P$ changes. Sea surface salinity and local $\delta^{18}O_w$ of seawater are directly linked to the $E-P$ balance^{18,19} and a linear correlation was found between these parameters for most of the global ocean¹⁹. The glacial to interglacial change in the oxygen isotope ratio for foraminifera ($\Delta\delta^{18}O_F$) can be expressed as follows²⁰:

$$\Delta\delta^{18}O_F = a + b\Delta T + c(S - S^*) \quad (1)$$

where $\Delta\delta^{18}O_F$ is the measured deviation between modern and past $\delta^{18}O_F$ values of planktonic foraminifera, a represents global variations of sea water $\delta^{18}O_{WG}$ (variable through time due to changes of the global ice volume), b is the slope of the $\delta^{18}O_F$ versus temperature relationship, ΔT is the local temperature variation, c is the slope of the $\delta^{18}O_w$ versus salinity relationship, S is the past local salinity and S^* is the modern local salinity augmented with the global salinity change due to changes of the global ice volume. From equation (1), we obtained S by the following equation:

$$S = S^* + (\Delta\delta^{18}O_F - a - b\Delta T)/c \quad (2)$$

The salinity S^* varies through time in response to the sea level variations (for example 120 m of sea level lowering during the Last Glacial Maximum (LGM) compared with a mean ocean depth of about 3,800 m). Consequently S^* can be expressed as:

$$S^* = S_0^* + (SL \cdot 35/3800) \quad (3)$$

where S_0^* is the modern local salinity and 35‰ the modern mean ocean salinity, SL = sea level changes expressed in m below modern level (lowering being counted as positive).

Therefore, the past local salinity can be estimated by the following equation:

$$S = S_0^* + (SL \cdot 35/3800) + (\Delta\delta^{18}O_F - a - b\Delta T)/c \quad (4)$$

In order to provide an index of local salinity variations due to the local changes of the $E-P$ balance, we also calculated ΔS values expressed as:

$$\Delta S = S - S^* = (\Delta\delta^{18}O_F - a - b\Delta T)/c \quad (5)$$

The seasonal range of SST is very small (about 1–2 °C) at the location of core MD900963^{11,21} and sediment trap studies^{22–24} demonstrate that the *G. ruber* bloom does not lag significantly the abrupt increases in wind speed which corresponds to the beginning of the monsoons. Consequently, it is reasonable to use the U_K SST record to obtain ΔT , and to apply temperature correction to the $\delta^{18}O_F$ record ($b = -0.2$ for living *G. ruber* in the Indian Ocean²⁵).

Labeyrie *et al.*²⁶ and Vogelsang²⁷ reconstructed a mean global seawater $\delta^{18}O_{WG}$ record for the past 200 kyr which is only affected by global ice volume changes. We normalized this seawater $\delta^{18}O_{WG}$ record to the global $\Delta\delta^{18}O$ value of 1.2‰, which has been established for the last deglaciation²⁸. To estimate local salinity variations, changes in the global seawater $\delta^{18}O_{WG}$ (term a in equation (2)) have to be removed from the SST corrected $\delta^{18}O_F$ record of MD900963. Since a point-by-point subtraction of two independent isotopic records is very sensitive to phase differences and thus to timescale details, we first correlated the global seawater $\delta^{18}O_{WG}$ reconstruction to the timescale of the temperature corrected isotope record of Core MD900963 before subtracting it (Fig. 2c, we used the ANALYSERIE software developed by D. Paillard & L. D. Labeyrie, CFR Gif-sur-Yvette).

In order to reconstruct the $\delta^{18}O_w$ versus salinity relationship (term c in equation (2)), we used 52 seawater $\delta^{18}O_w$ and salinity measurements between 20° S and 21° N from the Arabian Sea (J. C. Duplessy, personal communication). Because no seawater $\delta^{18}O_w$ measurements are available for the Bay of Bengal, a

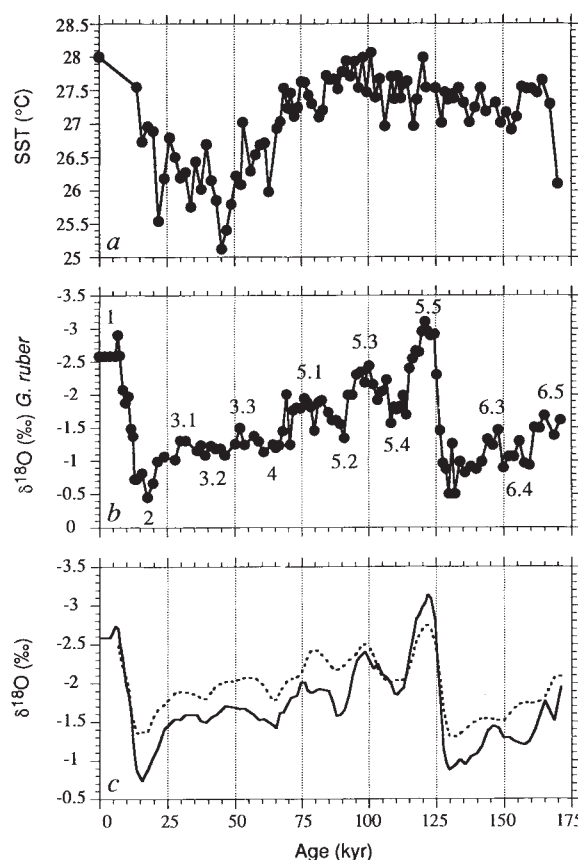


FIG. 2 SST (a) and $\delta^{18}O_F$ record (b) for Core MD900963. For missing core top SST and $\delta^{18}O_F$ values a constant modern annual mean SST of 28 °C (ref. 11) and a $\delta^{18}O$ value of -2.58 ‰ was used, assuming that sea level did not change during the last 6 kyr²⁸. c, SST corrected $\delta^{18}O_F$ record (solid line) in comparison with the global seawater $\delta^{18}O_{WG}$ record^{26,27} (broken line), correlated to the timescale of Core MD900963. Before calculation the raw data were smoothed using a least square procedure³¹.

$\delta^{18}O_w$ /salinity relationship was established by using 22 $\delta^{18}O_F$ *G. ruber* core top measurements⁷. To represent both data sets in the same graph (Fig. 3) we converted the $\delta^{18}O_F$ core top measurements into seawater $\delta^{18}O_w$ values by using the temperature equation of Duplessy *et al.*²⁵ established for living *G. ruber* in the Indian Ocean.

The $\delta^{18}O$ /salinity slope of 0.37 of the composite data set is probably the best value of c to be used in equations (4) and (5) to quantify the salinity variations at the location of Core MD900963. In order to illustrate the uncertainty associated with the conversion of $\delta^{18}O$ into surface salinity, we also used the individual slopes obtained for the two basins Arabian Sea and Bay of Bengal (dashed lines on Fig. 4a and 4b). Taking into account the standard reproducibility (± 1 sigma) on the SST (± 0.3 °C; ref. 17), on $\delta^{18}O_{WG}$ (± 0.07 ‰; ref. 29) and on $\delta^{18}O_F$ (± 0.05 ‰; ref. 30), it is possible to estimate an average analytical error of about ± 0.3 ‰ for the reconstructed Δ salinity values. This indicates that only the major trends of the S and ΔS records should be interpreted in term of palaeoceanographic variations.

Figure 4a shows the downcore ΔS changes (equation (5)) whereas Fig. 4b presents the corresponding S values which take into account the global salinity variation due to sea level changes (equation 4). In the following paragraph we will mainly discuss the ΔS changes which are only due to local variations of the $E-P$ balance.

Negative ΔS values of about -1 to -0.5 ‰ are observed between 125 and 115 kyr BP (stage 5.5) and between 9 and 6 kyr

(Holocene). Between 160 and 140 kyr (stages 6.4 and 6.3) and between 75 and 25 kyr (stages 5.0 to 3.1), ΔS is consistently positive with values ranging between 0.5 to 1‰. Three short periods of elevated ΔS are clearly present in the record with values higher than 1‰: 140 to 125 kyr (stages 6.2 to 6.0), 90 to 80 kyr (stage 5.2) and 20 to 15 kyr (stage 2).

Negative ΔS during stage 5.5 and the early Holocene indicate that $E - P$ was lower than today. Because the precipitation over

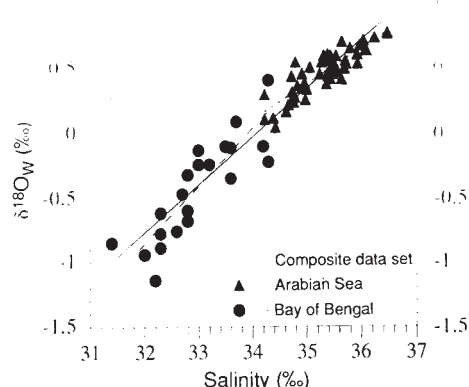


FIG. 3 $\delta^{18}\text{O}_w$ /salinity relationships for the Arabian Sea and the Bay of Bengal. Regression lines were calculated for these two basins and for the composite data set as follows: Arabian Sea, $y = -9.24 + 0.28x$ ($R = 0.87$); Bay of Bengal, $y = -15.20 + 0.45x$ ($R = 0.87$); composite data set, $y = -12.68 + 0.37x$ ($R = 0.95$). For this graph we used 52 seawater $\delta^{18}\text{O}_w$ and salinity measurements between 20°S and 21°N from the Arabian Sea (J. C. Duplessy, unpublished data). For the Bay of Bengal, a $\delta^{18}\text{O}_w$ /salinity relationship was established by using 22 $\delta^{18}\text{O}_F$ *G. ruber* core top measurements⁷. To represent both data sets in the same graph the $\delta^{18}\text{O}_F$ core top measurements were converted into seawater $\delta^{18}\text{O}_w$ values by using the temperature equation of Duplessy *et al.*²⁵ established for living *G. ruber* in the Indian Ocean, and by taking into account a $\delta^{18}\text{O}$ enrichment of about 0.85‰ due to late calcification²⁵.

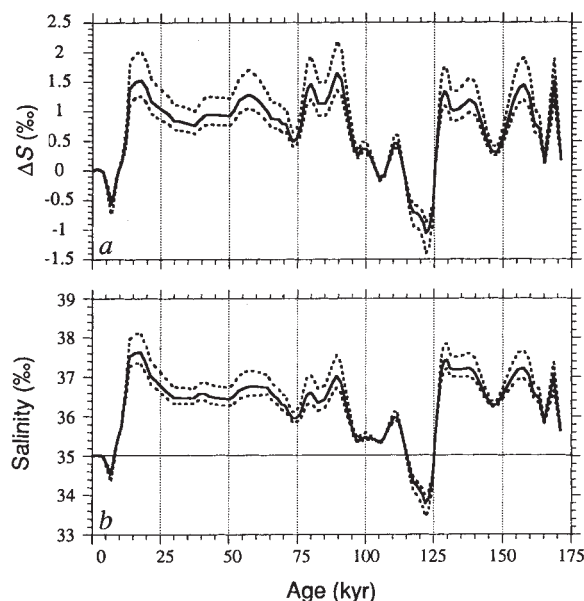


FIG. 4 a, Salinity variations ΔS for Core site MD900963. To eliminate high frequency variations due to the subtraction of two independent isotope records, we applied a least square filter to the salinity curves³¹. ΔS changes are calculated for the different slopes (C) of the $\delta^{18}\text{O}_w$ versus salinity relationships of the Arabian Sea (upper stippled line = 0.28), the Bay of Bengal (lower stippled line = 0.45) and the composite data set (solid line = 0.37). ΔS values are directly related to local variations of the $E - P$ balance. b, Local salinity variations S for Core site MD900963, taking into account salinity changes due to changes of global ice volume. The modern salinity of Core site MD900963 is taken as 35‰ (ref. 21).

India and the Indian Ocean is related to the SW monsoon strength³, the ΔS index suggests that the SW monsoon was stronger during these time intervals.

The higher ΔS values observed during glacial stages 6 and 2 and substage 5.2 on the Core site MD900963 were probably caused by increased evaporation and/or decreased precipitation. Consequently, it can be suggested that the dry NE monsoon was the dominating seasonal feature during these periods and/or that the humid SW monsoon was weakened. The generally high values obtained for the ΔS index (with the exception of stage 5.5 and the Holocene) suggest that in this part of the Indian Ocean the $E - P$ budget was different from the conditions of today during most of the last glacial cycle.

Based on pollen and foraminifera data^{4,6}, oxygen and carbon isotope composition of planktonic foraminifera^{5,8} and organic matter content⁸ similar climatic conditions were suggested for the LGM. During the LGM the decrease in precipitation led to considerably higher salinities in the Bay of Bengal due to the lower riverine freshwater input⁵. Our work extends these studies, and also shows that the Indian monsoon underwent large variations concomitant with the last global glacial-interglacial transition. Further SST determinations and salinity reconstructions are still necessary for the two basins (Arabian Sea and Bay of Bengal) both in order to calculate and model the changes of E and P independently and to understand in detail the climatology of the northern Indian Ocean during glacial periods. It will also be important to study the time variations of the marine productivity in the monsoonal upwelling zones off Somalia and Arabia, and their relationships with the salinity index reconstructed in this paper. □

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Century-scale events in monsoonal climate over the past 24,000 years

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BOTH the marine sediment record and numerical modelling of the atmospheric summer circulation over the northern Indian Ocean and southeast Asia have shown that the monsoonal climate exhibits a direct but nonlinear response to the intensity of solar insolation during summer, with a time lag of several thousand years^{1,2}. Here we present evidence from a high-resolution record of oxygen isotopes and carbonate spanning the past 24,000 calendar years that the response of the southwest monsoon over the Arabian Sea to long-term, gradual insolation changes occurred in several distinct events of less than 300 years duration, at 14,300, 13,500, 13,060, 9,900, 8,800 and 7,300 ¹⁴C yr BP. Thus, during this transitional period from glacial to post-glacial conditions the slow solar forcing seems to have induced very rapid changes in local climate. We speculate that the rapid response may be related to albedo changes in Asia.

Our objective was to monitor the chronology of the evolution of the monsoonal system over southeast Asia since the last glaciation. The Arabian Sea is especially suited to this because its deep-sea sediments permit extraction of continuous records of wind-driven oceanic upwelling, continental humidity, and dust and river discharge.

We sampled core 74KL from the upwelling region of the western Arabian Sea (Fig. 1) at 2.5-cm intervals, which corresponds to an average time resolution of 300 calendar years (Table 1). The core was obtained from the East Sheba Ridge 1,000 m above the turbidite plain of the Indus fan, 300 km south of the Arabian continental margin. Sediment structures suggest that the record of the uppermost 2 m of core 74KL is continuous and undisturbed. The stratigraphic base of 74KL is the $\delta^{18}\text{O}$ record of the surface-dwelling planktonic foraminifera *Globigerinoides*

ruber. Its oxygen isotope composition decreased from glacial values near 0‰ to Holocene values averaging near 1.75‰ (Fig. 2), punctuated by a series of abrupt changes. Of this range, 1.2‰ can be explained by changes in the global ice volume^{3,4}, but 0.55‰ should be of local origin. This local contribution must be reflected in the abrupt events, because these are of far larger amplitude than can be accounted for on the basis of changes in the global $\delta^{18}\text{O}$ value of sea water owing to ice-sheet melting. For example, the 0.5‰ change during event 5 is larger by 0.35‰ than the global increase of 0.15‰ owing to the meltwater pulse 1b that occurred at this time⁵.

To verify that the abrupt changes observed in core 74KL are not confined only to the upwelling areas off Arabia, we compared them with high-resolution records of cores KS8 and 422 from the Gulf of Oman, 900 km further north (Fig. 1). These also show the $\delta^{18}\text{O}$ events 2, 3, and 5, and a decrease in both grain sizes and wind-borne lithogenic carbonate content during event 3.

These abrupt shifts towards lighter $\delta^{18}\text{O}$ values in all three cores can be related to four possible mechanisms: (1) increased precipitation or decreased evaporation, leading to lower salinities and lower $\delta^{18}\text{O}$ values in the Arabian Sea surface water⁶; (2) an increased proportion of isotopically light Persian Gulf outflow water ($\delta^{18}\text{O}$: 0.7‰ SMOW at 200 m depth) and Red Sea outflow water ($\delta^{18}\text{O}$: 0.25–0.4‰ SMOW, at 200–1,200 m depth in the outer Gulf of Aden) that are admixed to the isotopically enriched South Indian Central Water ($\delta^{18}\text{O}$: 1.3‰ SMOW, 100–200 m depth) and Arabian Sea surface waters ($\delta^{18}\text{O}$: 0.9‰ SMOW at 0–100 m depth)^{7–9}; (3) a change in the seasonality of *G. ruber* growth, which today reveals two seasonal maxima of production, one with more positive values during the nutrient-rich summer months and a second one with more negative values in the warm winter surface waters^{10,11}; (4) an increase in sea surface temperature (SST). This change, however, seems unlikely, because it would imply warmer SST during the Holocene, when upwelling was increased and SSTs were colder than during the glacial period¹².

Which of these possible explanations is the actual cause for the abrupt steps towards lighter $\delta^{18}\text{O}$ values cannot be resolved at this stage. However, in every case, these changes occurred within less than 300 years (in fact, within less than 120 years in core 422; FS manuscript in preparation) and abrupt transitions are found at 13,500; 13,060; 9,900; 8,800; and 7,300 ¹⁴C yr BP (Fig. 2). The $\delta^{18}\text{O}$ decrease at 12,000 ¹⁴C yr BP is only a spike and does not qualify for an event, which we define as a change from one sample to another, reaching a new level of similar values. All of the abrupt steps in the $\delta^{18}\text{O}$ record can be regarded as

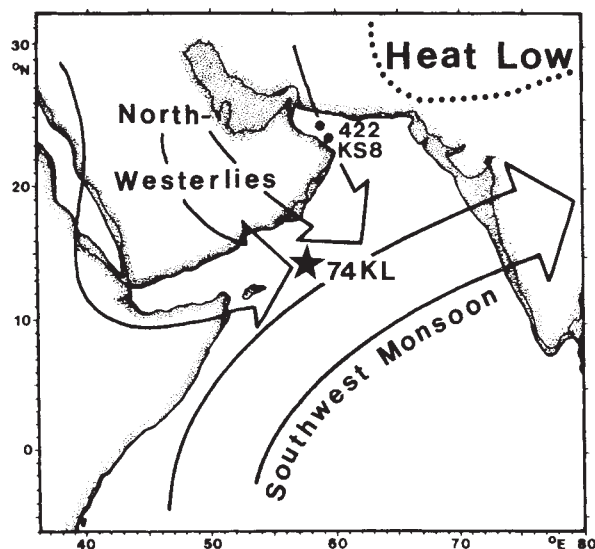
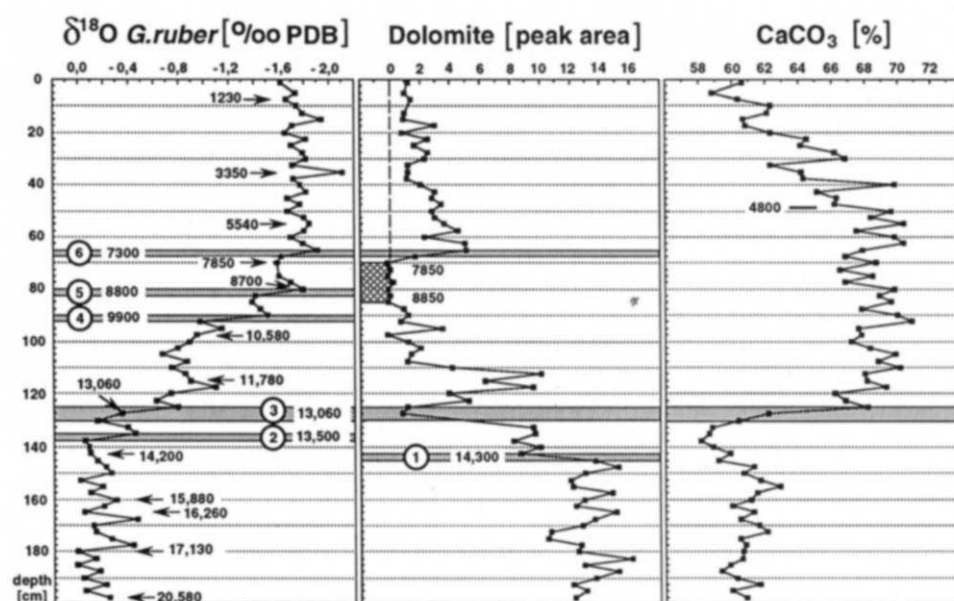


FIG. 1 The Arabian Sea during summer season. A thermal heat low over Asia attracts rain-bearing southwesterly winds from the Southern Hemisphere and dry northwesterlies from Arabia, the northern branch of which carries huge amounts of dolomite (up to 60% of total dust load in aerosol samples in Kuwait)³⁴. Stippled areas indicate dry land during glacial times of lowered sea level. Sediment cores: 74KL (14° 19.26' N, 57° 20.82' E, 3,212 m); KS8 (23° 28.00' N, 59° 11.50' E, 2,900 m); 422 (24° 23.40' N, 59° 02.50' E, 2,732 m).

FIG. 2 AMS radiocarbon ages of core 74 KL (Table 1) and $\delta^{18}\text{O}$ values of *G. ruber*, diameter: 315–400 μm , 20 specimens, cleaned in methanol and ultrasonic bath, measured at the ^{14}C laboratory of Kiel University. Dolomite was measured with a Philips diffractometer at 2.89 Å in the lithogenic fraction > 2 μm . Values represent the peak area from 35.7° to 36.5° 2 θ (Co K α) and can be regarded as being comparable to percentage (compare percentages in refs ^{13, 14}). The sharp increase in CaCO_3 content at 130–125 cm is most probably caused by a reduction of lithic components, the flux of which we quantified as 43 $\text{g m}^{-2} \text{yr}^{-1}$ during the last glacial and 18 $\text{g m}^{-2} \text{yr}^{-1}$ during the Early Holocene and 29 $\text{g m}^{-2} \text{yr}^{-1}$ during the Late Holocene (F.S., manuscript in preparation). The hatched area between 85 cm and 70 cm depth indicate the depth interval of the Early Holocene southwest monsoon maximum, whereas the short line at 50 cm depth indicates the knickpoint (change in profile) towards the Late Holocene aridification.



being of mostly local origin, because the glacial/interglacial contrast in distribution patterns of $\delta^{18}\text{O}$ (ref. 6) had revealed large local amplitudes, and there are no other records worldwide to show the same succession of events.

Beside the hydrographic events, we observe changes of similar abruptness in the lithic sediment supply, which is exclusively of aeolian origin in the western Arabian Sea², stemming from dust plumes raised in Arabia and Mesopotamia during spring and summer, and transported to the Arabian Sea by northwesterly winds (Fig. 1)^{13,14}. The provenance of the lithic aerosol fraction in these dust plumes is best recorded by the occurrence of dolomite, which is preferentially transported in

the northern branch of the northwesterlies, because dolomite is derived from Mesozoic strata that are prominent only in northern Arabia, or from sebkha sediments around the Persian Gulf, characterized by the frequent occurrence of protodolomite. The CaCO_3 content of the Arabian Sea sediments represents another record of lithic sediment supply, because the carbonate percentages in the open ocean at less than 3,500 m water depth are largely controlled by dilution with lithic matter^{2,14}.

The northwesterly winds that carry these dust loads and also the southwest monsoon are both attracted by the Asian heat low in such a way that the southward (eastward) extent of the

TABLE 1 ^{14}C AMS ages

AMS sample depth (cm)	AMS ^{14}C age (^{14}C yr BP)	Error 1-sigma (yr)	Calibrated age (cal. yr BP)	Reference for calibration	Event	Event depth (cm)	Calibrated event age (cal. yr BP)	Interpolated event age (^{14}C yr BP)
7.50	1,230	60	1,170	30				
35.00	3,350	100	3,632	31				
55.00	5,540	90	6,320	31	Aridification	48.75	5,500	4,800
70.00	7,850	90	8,625	32	Event 6: Humid interval	66.25	8,050	7,300
80.00	8,700	120	9,550	33		70.00	8,600	7,850
97.50	10,580	120	11,880	33	Event 5: Humid interval	81.25	9,700	8,800
115.00	11,780	130	13,780	28	Event 4:	82.50	9,900	8,850
127.50	13,060	150	16,060	28		91.25	11,450	9,900
142.50	14,200	170	17,700	28	Event 3: Event 2:	127.50	16,060	13,060
160.00	15,880	170	19,380	28		136.25	17,000	13,500
165.00	16,260	170	19,760	28	Event 1:	143.75	17,800	14,300
180.00	17,130	180	20,630	28				
197.50	20,580	260	24,080	28				

Dating: ^{14}C AMS ages of core 74KL, analysed on 10 mg of *G. ruber*, diameter 315–400 μm , at the Centre des Faibles Radioactivités, CNRS, Gif sur Yvette, France. A 400-yr correction is applied for the age of the sea water. Direct measurements²³ of the age of sea water in the coastal upwelling water of Oman revealed ages of 800 yr, so our use of a 400-yr correction has some uncertainties. Carbon-14 ages for events were interpolated from the U/Th-corrected ^{14}C AMS age profile and then reconverted to the conventional ^{14}C scale^{28,29} using the switch points at 9,100 ^{14}C yr = 9,800 calendar yr BP; 10,400 ^{14}C yr = 12,400 calendar yr BP; and 13,100 ^{14}C yr = 15,100 calendar yr BP, 13,200 ^{14}C yr = 16,700 calendar yr BP.

northwesterlies outlines the northernmost (westward) extent of the southwest monsoon winds¹⁵ (Fig. 1). High dolomite flux thus indicates a weak southwest monsoon and low dolomite indicates a strong southwest monsoon. To complicate the picture, on glacial/interglacial scales the dolomite supply must also be affected by sea-level changes, because the Persian Gulf was dry land during glacial times. It was most probably a dust-entrainment area, which was lost during the transgression into the Gulf^{14,16}.

The initial onset of climate change in Southeast Asia at the end of the glacial is marked by event 1, 14,300 ¹⁴C yr BP. This decrease of dolomite content must have been due to changes in the atmospheric circulation, because sea level was still 110 m below the present level and therefore well below the transgression level into the Gulf (100–65 m below modern surface waters)¹⁶. During event 3 (13,060 ¹⁴C yr BP), which represents the major transition between the glacial and the Holocene, however, we observe a simultaneous sharp change of $\delta^{18}\text{O}$, dolomite and CaCO_3 content.

A brief climatic extreme is recorded by the total absence of dolomite dust input from 8,850–7,850 ¹⁴C yr BP, when dust plumes from the Persian Gulf region did not reach as far south as our core position. The continental heat low was strongest during this time¹ and the track of the southwest monsoon and its associated pattern of precipitation can be expected to have shifted to their northernmost position. Indeed, there was widespread moisture all over East Africa, Arabia, Pakistan, northern India, and even Tibet, characterizing an early Holocene humid interval^{17–21}. This indicates that the convergence between the southwest monsoon and the northwesterlies, which occurs mostly over the ocean today, was then located over inner Arabia. Judging from the actual age of the humid interval (9,900–8,600 calendar yr BP, Table 1), it followed immediately after the maximum summer insolation at 20° N to 35° N, 10,000 calendar years ago²².

The middle Holocene was then characterized by the return of dolomite after 7,300 ¹⁴C yr BP (event 6) and a general increase in dust flux (decrease in % CaCO_3) after 4,800 ¹⁴C yr BP, equal to 5,500 calendar yr BP, which indicates a middle Holocene increase in Arabian aridity. This deterioration is reflected all over North Africa and Arabia by lowered lake levels^{17,20,21}, a decline of vegetation cover^{18,19} and, especially, the formation of hierarchical societies in the then overpopulated Nile valley and Mesopotamia starting near 5,300 calendar yr BP. In contrast, neolithic settlements in the inner desert of Arabia have been

completely abandoned between 5,600 and 5,200 calendar yr BP²³.

To infer the mechanisms that caused these abrupt transitions in the monsoonal system, we focus on the largest event of atmospheric and hydrographic change, event 3 (13,060 ¹⁴C yr BP). This was at the very beginning of global meltwater pulse 1a, which started at 13,000 ¹⁴C yr BP and culminated about 12,300 ¹⁴C yr BP ago⁵. One possible mechanism that could explain such an early monsoon response is a reaction to albedo changes in Asia during the initial phases of deglaciation, when areas of snow fields at high elevation disappear and expose the soils to the slowly increasing solar radiation. This mechanism would be fast enough to trigger abrupt climate changes. Its effect would be a sharp increase in the length of the summer season, with effects on the seasonal patterns of wind trajectories as well as on the strength and duration of the upwelling season.

Sensitivity experiments with global circulation models have indeed shown that albedo changes induced by changing snow cover exert considerable control over the development of the continental heat low over mainland Asia, and affect the strength and duration of the southwest monsoon winds over the Arabian Sea^{1,24,25}. Accordingly, a synchronous evolution of deglaciation events and southwest monsoon intensification should be physically related. Modern meteorological data reveal that in general a cold winter over the Northern Hemisphere is followed by a weakened heat low and weakened southwest monsoon intensity during the next summer²⁶.

Time-series work on monsoonal change over the past 350,000 years demonstrates that the response of atmospheric circulation, coastal upwelling, and continental aridity lagged the changes in solar forcing by several thousand years². The succession of atmospheric and hydrographic events in the Arabian Sea shows, however, that these transitions are complicated and are observed not as a gradual response in each part of the climate system, but as a number of very brief evolutionary steps, interdependent in the atmosphere and the ocean.

There might, however, be a stable time-related pattern in the succession of events. When converted to a calendar year timescale (Table 1), the interval between events 5–6, 4–5 and 1–3 lasted about 1,700 years. This recurrence period may be characteristic of the internal variability in the monsoon region; a similar period was found in the $\delta^{13}\text{C}$ record of *G. ruber* in core 74KL, and is also known from SST variations in the East Atlantic upwelling belt²⁷. □

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Isotopic comparison of K/T boundary impact glass with melt rock from the Chicxulub and Manson impact structures

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THE discussion of candidate source craters for the catastrophic impact that occurred at the boundary between the Cretaceous and Tertiary periods (K/T boundary) has recently centred on two buried craters: the Chicxulub in Mexico (>200 km diameter) and the Manson in Iowa (~35 km diameter), both of which have ^{40}Ar – ^{39}Ar ages of 65 Myr, indistinguishable from that of impact glass spherules found in K/T boundary sediments^{1–4}. Here we report the strontium, neodymium and oxygen isotopic compositions of core samples of impact melt rock recovered from drill holes into both the Chicxulub and Manson craters, and compare these with previously published isotopic data for impact glasses from the K/T boundary of the Beloc Formation in Haiti^{5–7}. The Chicxulub melt rocks are isotopically indistinguishable from the K/T impact glass, strongly supporting the proposition that Chicxulub is a source crater for the K/T catastrophe. The Manson melt rocks, by contrast, have a clearly different isotopic composition, strongly suggesting that they are unrelated to the K/T impact spherules.

Since the original proposition by Alvarez *et al.*⁸ that the K/T boundary was marked by a giant impact which led to the extinction of over half the species on Earth, researchers have been searching for the source crater for a large K/T impact event^{9–14}. Identification of impact-glass spherules (and fragments) at the K/T boundary in Haiti provided the first unambiguous sampling of the elemental and isotopic composition of at least part of the target material^{5–7,15}. Oxygen isotope studies of the impact glasses showed that they were formed by impact-induced mixing of a siliceous target rock endmember with a mixed carbonate–evaporite rock endmember, but as both

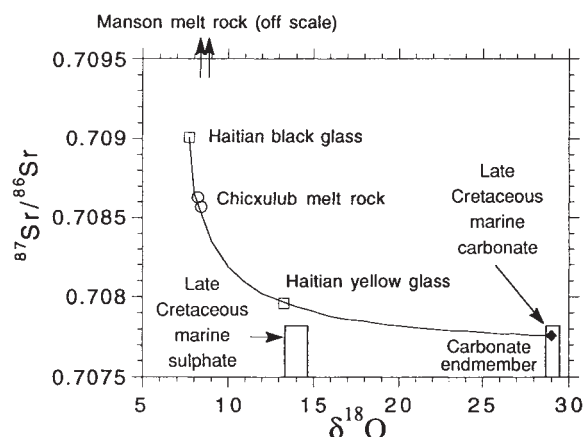


FIG. 1 Plot of $^{87}\text{Sr}/^{86}\text{Sr}$ at the time of impact (65 Myr ago) against $\delta^{18}\text{O}$ for Haitian black and yellow impact glass⁵ (squares), Chicxulub melt rock (circles), Manson melt rock (off scale), a calculated carbonate endmember discussed in the text (diamond) and the range of values for Late Cretaceous marine sulphate²⁴ and carbonate¹⁶. A mixing curve that was calculated for mixtures of average Haitian black glass and the carbonate endmember is also shown. The Manson melt-rock samples have $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of 0.722350 and 0.724710, which differ from the Haitian black glass by ~8 times more than the total isotopic variation displayed on the diagram. Uncertainties are smaller than symbol sizes.

of the candidate craters have this combination of rock in their target stratigraphy, this did not provide a means for choosing between them⁷. High-precision ^{40}Ar – ^{39}Ar dating of Haitian K/T impact glasses and of melt rock recovered from drill holes into both the Chicxulub and Manson structures have also been unable to distinguish the source crater because all three are of identical age within the analytical uncertainties^{1–4}.

We have measured the strontium, neodymium and oxygen isotopic composition of whole-rock samples of melt rock which show no petrographic or isotopic signs of alteration (see Table 1), recovered from the C-1 drillhole into the central peak of the Chicxulub crater (C1-N10-1A and C1-N10-2)³ and the M-1 drillhole into the central peak of the Manson crater (M1-359.6 and M1-475.3)¹². Samples from Chicxulub were recovered from 1,393 to 1,394 m below sea level and are fine- to medium-grained melt rock composed of pyroxene, feldspar, quartz and opaques. Samples from Manson were recovered 109.6 and 144.9 m below the surface and are fine-grained recrystallized 'glassy' matrix within a crystalline rock breccia.

Haitian glass is highly variable in composition and falls into

TABLE 1 Isotopic data

Sample	$^{87}\text{Sr}/^{86}\text{Sr}$ (meas)	$^{87}\text{Sr}/^{86}\text{Sr}$ (65 Myr)	ϵ_{Sr} (65 Myr)	Sr (p.p.m.)	Rb (p.p.m.)	$^{143}\text{Nd}/^{144}\text{Nd}$ (meas)	$^{143}\text{Nd}/^{144}\text{Nd}$ (65 Myr)	ϵ_{Nd} (65 Myr)	Nd (p.p.m.)	Sm (p.p.m.)	$\delta^{18}\text{O}$ ‰
C1-N10-1A	0.709102(31)	0.708634	59	324	56.8	0.512449(20)	0.512395	-3.1	20.0	4.25	8.2
C1-N10-2	0.709024(31)	0.708570	58	299	50.8	0.512465(29)	0.512410	-2.8	19.6	4.17	8.4
M1-359.6	0.723847(28)	0.722349	253	223	125	0.511984(30)	0.511931	-12.2	31.2	6.43	8.9
M1-475.3	0.726514(28)	0.724717	287	229	154	0.511912(29)	0.511857	-13.6	26.1	5.54	8.7

Isotopic compositions of Sr, Nd and O and concentrations (in p.p.m.) of Sr, Rb, Nd and Sm in drill-core samples of melt rock from the Chicxulub and Manson impact structures. Sr and Nd isotopic compositions were measured by thermal ionization mass spectrometry after clean-laboratory digestion in HF-HClO_4 and chemical separation in quartz cation exchange columns. Sr and Nd isotopic compositions are given as measured and corrected for 65 Myr of radioactive decay. ϵ_{Sr} and ϵ_{Nd} are the deviations in the $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in parts per 10⁴ from the unfractionated mantle reservoir value of 0.7045 and the chondritic uniform reservoir value of 0.512638, respectively. Measured Sr and Nd isotopic compositions represent the means of 150 to 200 ratios; 2σ uncertainties are given in parentheses for the last two decimal places. The Sr isotopic compositions were normalized to $^{84}\text{Sr}/^{86}\text{Sr}=0.1194$ and the Nd isotopic compositions were normalized to $^{146}\text{Nd}/^{144}\text{Nd}=0.7219$. Total procedural blanks were <20 pg Nd and <42 pg Sr and are negligible. Uncertainties of isotope dilution concentration measurements for Sr, Nd and Sm are ~0.2% and for Rb are ~0.5%. $\delta^{18}\text{O}$ values are the deviation in the $^{18}\text{O}/^{16}\text{O}$ ratio in permil from the value of standard mean ocean water and have uncertainties of ~0.1‰. $\delta^{18}\text{O}$ values of quartz (10.2‰), plagioclase (8.0‰) and pyroxene (7.2‰) from Chicxulub melt rock show that the minerals were in isotopic equilibrium at ~700 °C and have not undergone subsolidus hydrothermal alteration.

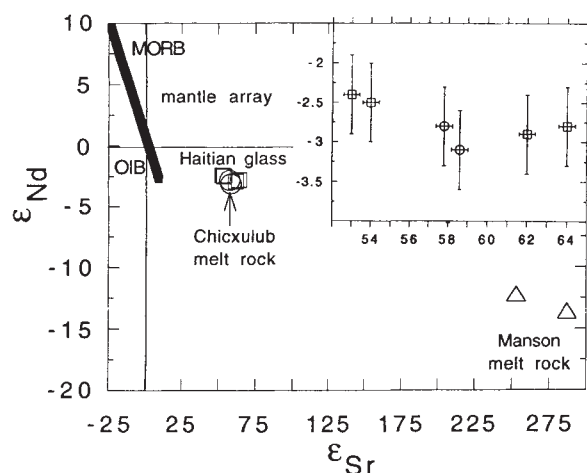


FIG. 2 Plot of ϵ_{Nd} against ϵ_{Sr} for samples of Haitian black glass⁶ (squares) and melt rock from the Chicxulub (circles) and Manson (triangles) impact structures at the time of impact 65 Myr ago. The ϵ notation is defined in Table 1. Inset shows the details of the Haitian and Chicxulub data at an expanded scale. The mantle array follows a trend between the mid-ocean-ridge basalt (MORB) and ocean-island basalt (OIB) fields and is shown to emphasize the significant difference in the isotopic composition between mantle-derived volcanic rocks and the Haitian glass and Chicxulub melt rock. A previous isotopic analysis of pervasively altered melt rock from the drill hole Y-6 into the Chicxulub structure¹¹ yielded a similar ϵ_{Sr} (65 Myr) value of +55 and a significantly different ϵ_{Nd} (65 Myr) value of -5.0. The inconsistency in the ϵ_{Nd} (65 Myr) value may be due either to secondary anhydrite, calcite and quartz veins present in the Y-6 sample or isotopic heterogeneity in the melt sheet. Uncertainties are smaller than symbol sizes except in the inset where error bars are shown.

two general compositional ranges: the abundant high-SiO₂ black glass, and the less abundant (~2% of total glass) high-CaO yellow glass. The low abundance of high-Ca yellow glass may reflect its more rapid alteration in sediment. In a previous study⁷, we considered the major element (and sulphur) concentrations and the oxygen isotopic compositions of the Haitian glasses and concluded through mixing calculations that the glasses formed by impact-induced mixing of ~58% silicate rock (with the average composition of the black glass) with ~42% platform carbonate (containing <10% sulphate-rich evaporite). From our mixing model⁷ and the Sr and O isotopic composition of the average black and yellow glass, we can calculate a Sr isotopic composition of a hypothetical platform carbonate endmember which, when mixed in the above proportion with average black glass, will yield a Sr and O isotopic composition identical to that of the yellow glass. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the carbonate endmember calculated above is 0.70776, which coincides with the range of values for Late Cretaceous marine carbonates¹⁶ (Fig. 1). Such a mixture of silicate (low Sr concentration) with carbonate (high Sr concentration) will form a mixing curve when $^{87}\text{Sr}/^{86}\text{Sr}$ is plotted against $\delta^{18}\text{O}$ (Fig. 1)¹⁷.

If impact melts from either Chicxulub or Manson formed by mixing of the same (or closely related) endmembers that formed the Haitian glasses, we would expect them to plot on the mixing curve in Fig. 1. The two melt rock samples from Chicxulub plot precisely on the mixing curve close to the Haitian black glass endmember (Fig. 1), whereas the two melt-rock samples from Manson have extremely high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios plotting far above the curve and suggesting that they are unrelated to the Haitian glass. The position of the Chicxulub samples on the mixing curve in Fig. 1 indicates that they could be formed by mixture of 94% black glass with 6% platform carbonate. Although we have used a silicate endmember of average black glass composition (64%

SiO₂), we reiterate that the black glass ranges considerably in elemental and isotopic composition because it can contain up to 20% of the carbonate endmember⁷. The major element^{3,11} and Sr and O isotopic composition (Table 1) of the Chicxulub melt rock is identical to some of the more Ca-rich samples of Haitian black glass that we⁷ and others^{5,6} have analysed and is consistent with our mixing model.

In addition to the single Sr and O isotope analysis of Haitian black glass by Sigurdsson *et al.*⁵ (Fig. 1), Premo and Izett⁶ measured the Sr and Nd isotopic compositions of four samples of Haitian black glass. They observed considerable variability in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio but no significant variability in the $^{143}\text{Nd}/^{144}\text{Nd}$ ratio. On Fig. 2 we present these data as well as our data for the Chicxulub and Manson melt rocks on a plot of ϵ_{Sr} against ϵ_{Nd} (the ϵ notation is defined in Table 1). The Chicxulub melt rock has a Nd isotopic composition indistinguishable from that of the Haitian glasses, and a Sr isotopic composition intermediate between those of the various samples of Haitian glass (inset on Fig. 2), further supporting the genetic link between these samples. The Manson melt-rock samples have highly contrasting Sr and Nd isotopic compositions again suggesting that they are not related to the Haitian glass.

The Chicxulub melt rock and Haitian impact glasses could have been derived from mixing of carbonate either with siliceous sedimentary or meta-sedimentary rocks, or with igneous rocks of andesitic or granodioritic composition. Depleted mantle Nd model ages, which are related to the average crustal residence time of the source (or sources) of the target material, may be useful indicators of the provenance of the impact melt source materials^{6,18,19}. Nd model ages for the Chicxulub melt rock and Haitian glass fall in the narrow range of 1,040 to 1,080 Myr, suggesting that the silicate endmember of the target stratigraphy had a source with a middle Proterozoic average crustal residence age. The sedimentation or igneous crystallization age of the putative silicate endmember is almost certainly younger than the ~1060 Myr average crustal residence age. Therefore, shocked-zircon U-Pb ages of ~330 to 575 Myr from the K/T boundary^{20,21} are not inconsistent with the Nd model ages presented here.

Because the $\delta^{18}\text{O}$ values for the high-SiO₂ Haitian glasses and Chicxulub melt rock (6.0 to 9.0‰ and 8.2 to 8.4‰, respectively)⁷ are in the range of igneous (but not sedimentary) values, we favour the idea that carbonate was mixed with andesitic or granodioritic basement rock during impact. However, as previously pointed out⁷, we cannot rule out the possibility that carbonate was mixed with water-saturated siliceous sedimentary rock, and that the $\delta^{18}\text{O}$ value of the rock was lowered from a higher sedimentary value during impact because of admixture of meteoric water.

The close genetic link that has been forged between the Haitian impact glass and Chicxulub melt rock may have important implications for understanding the ejection of melts during impacts⁶. In the case of the Australasian and moldavite tektite-strewn fields there is evidence that the tektites originated from the uppermost ~200 m of strata^{18,19}. In the case of the Haitian impact glasses, however, it appears that the ejected material might have come from the Chicxulub crater melt sheet, or at least was derived from very similar target stratigraphy. One possibility is that the impact caused mixing of Late Cretaceous carbonates with underlying silicate basement (at a depth of ~3 km; ref. 22). If it is confirmed that there is a difference between the depth of origin of Australasian and moldavite tektites and melts ejected during the K/T impact, this may yield information on the relationship between crater scaling laws²³ and the ejection of impact melts. □

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Expression of the UNC-5 guidance receptor in the touch neurons of *C. elegans* steers their axons dorsally

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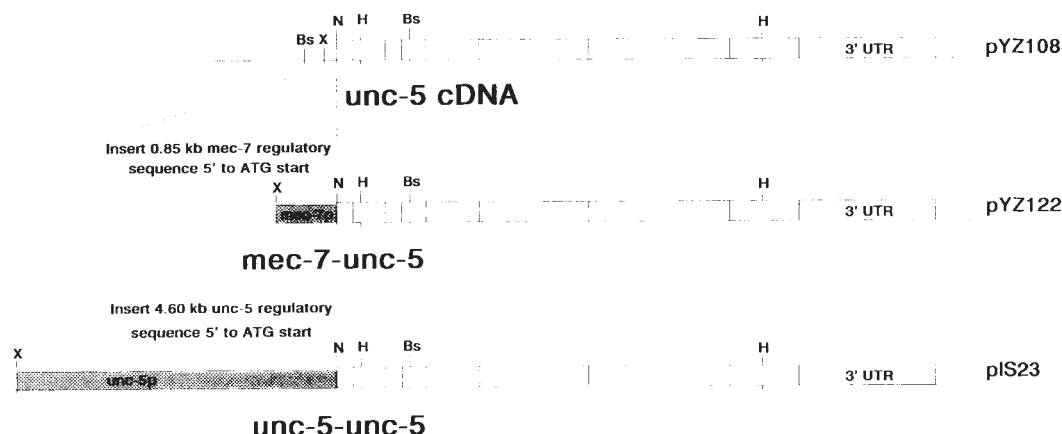
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GROWTH cones in developing nervous systems encounter a sequence of extracellular cues during migration^{1,2}. In theory, a growth cone can navigate by selectively expressing or activating surface receptor(s) that recognize extracellular cues appropriate to each migratory phase. Using the simple *Caenorhabditis elegans* nervous system, we attempted to demonstrate that path selection by migrating growth cones can be predictably altered by ectopic expression of a single receptor. The *unc-5* gene of *C. elegans* encodes a unique

receptor of the immunoglobulin superfamily (UNC-5), required cell-autonomously to guide growth cone and mesodermal cell migrations in a dorsal direction on the epidermis^{3,4}. We report here that the UNC-5 receptor induces dorsally oriented axon trajectories when ectopically expressed in the touch receptor neurons which normally extend pioneer axons longitudinally or ventrally on the epidermis⁵. These errant trajectories depend on *unc-6*, which encodes a putative epidermal path cue⁶, just as normal dorsally oriented axon trajectories do (such as those of certain motor neurons⁴), suggesting that UNC-5 acts to reorient the touch cell growth cones by using its normal guidance mechanisms. These results support previous evidence that UNC-5 and UNC-6 play instructive rules in guiding growth cone migrations on the epidermis in *C. elegans*⁴, and indicate that pioneering growth cones, which normally migrate in different directions, may use equivalent intracellular signalling mechanisms for guidance.

A *lacZ* reporter fused to the 5'-flanking sequence of the *mec-7* gene is expressed specifically and abundantly in the six touch receptor neurons⁷. We used the same *mec-7* regulatory sequences to drive *unc-5* expression in these cells. The touch receptor neurons have appropriate trajectories for testing the effects of ectopic expression of UNC-5 on axon guidance: axons of neurons ALML/R and PLML/R extend anteriorly along the lateral surface of the body wall, whereas those of AVM and PVM first extend ventrally on the body wall, then anteriorly along the ventral nerve cord⁵. These neurons do not express *unc-5* (refs 3, 4).

FIG. 1 Plasmid constructs carrying an *unc-5* cDNA under the control of the *mec-7* or *unc-5* 5' flanking regulatory sequence. 0.85 kilobase (kb) (*mec-7p* shaded) or 4.6 kb (*unc-5p* shaded) regulatory sequences immediately 5' to the translation initiation codons of *mec-7* or *unc-5*, respectively, were inserted immediately 5' to the initiation codon in exon 2 (ref. 3) of a 3.6 kb *unc-5* cDNA (exons and a 3' untranslated region (3'UTR) are indicated as adjacent unshaded rectangles). The resulting *unc-5-unc-5* and *mec-7-unc-5* constructs were injected into adult gonads of *unc-5(e53)* mutant and wild-type animals, respectively, with *mec-7-lacZ*, and either of the co-transformation marker DNAs that induce a Roller or Twitcher phenotype (described in Fig. 2 legend). The *unc-5-unc-5* construct stably rescued the Unc (uncoordinated) and Dtc (distal tip cell) phenotypes of *unc-5(e53)* self-fertilization progeny. The possible rescue of other *unc-5(e53)* migration defects⁴ was not analysed in these transformants. The *unc-5* cDNA in plasmid pYZ108 was assembled from several sources, including a PCR product made with a 5' primer that introduced an NcoI



(N) site at the predicted ATG initiation codon at the 5' end of exon 2 (ref. 3) without changing the coding sequence. All components (PCR products and partial cDNAs) used to assemble pYZ108 were sequenced in their entirety. The 5' flanking regulatory regions of *mec-7* and *unc-5* were mutagenized by PCR using a 3' primer which introduced an NcoI site into the ATG translation initiation codon of each gene and a 5' primer. These 0.85 kb (*mec-7p* shaded) and 4.6 kb (*unc-5p* shaded) XbaI–NcoI fragments were cloned into pYZ108 to derive *mec-7-unc-5* (pYZ122) and *unc-5-unc-5* (pIS23) plasmids, respectively.

The construct used to express *unc-5* in the touch neurons (Fig. 1) contained a full-length *unc-5* complementary DNA linked to the 5'-flanking sequence of *mec-7*. The *unc-5* cDNA encodes functional UNC-5 receptor, because it can rescue the *unc-5* mutant phenotype when linked to the 5' regulatory sequences of the *unc-5* gene itself (Fig. 1 legend). We injected the *mec-7-unc-5* plasmid together with the *mec-7-lacZ* construct described earlier, and one of two marker DNAs. Transformants were identified by virtue of phenotypic expression of the marker. Those that founded the lines used in this study transmitted the injected DNAs as an extrachromosomal element⁸. Histochemical staining for β -galactosidase activity allowed the trajectories of the touch neuron axons to be observed⁷.

Nearly all touch cell axons in *mec-7-unc-5* transgenic animals extend dorsally, instead of along their normal wild-type longitudinal (ALML/R and PLML/R) or ventrally oriented (AVM,

PVM) paths (Figs 2 and 3). The trajectories that these errant axons take to the dorsal side vary occasionally (Fig. 3). Most AVM (34/46) and PVM (58/68) axons grow in a straight dorsal direction, sometimes making a posterior longitudinal excursion before doing so (8/114) (Fig. 3). Most ALM (49/95) and PLM (90/163) axons grow obliquely to reach the dorsal side. All of the touch cell axons turn along a longitudinal path at the dorsal limit of their trajectory. About half of the *mec-7-unc-5* transgenic animals are touch-insensitive, consistent with their errant touch cell axon trajectories and cell body displacements (see below).

Minor cell body displacements also occur in *mec-7-unc-5* transgenic animals. ALM and PLM cell bodies are sometimes on the dorsal side. PLM, ALM and AVM cell bodies are occasionally displaced anteriorly and PVM cell bodies are occasionally displaced posteriorly (Fig. 3). In the wild type, ALM and AVM/PVM ancestors migrate long distances embryonically and

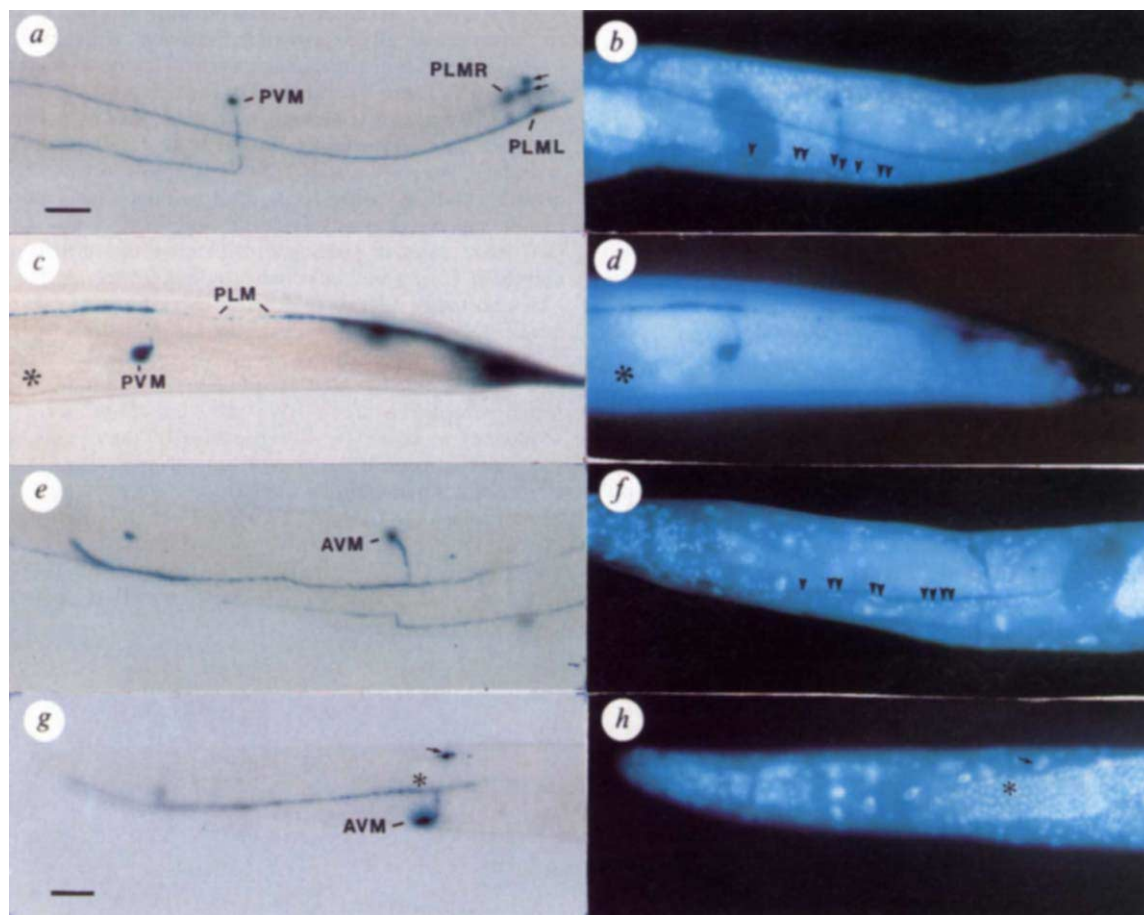


FIG. 2 The *mec-7-unc-5* transgene induces dorsally oriented trajectories in the touch neurons. In all panels, dorsal is up, ventral is down, and anterior is to the left. Animals are double-stained for β -galactosidase activity (left side) and with DAPI to visualize nuclei (right side). The control animal in a and b is transgenic for the extrachromosomal array evEx1 derived from germ-line co-transformation with pNW115 (*mec-7-lacZ* to allow expression of β -galactosidase in the touch neurons⁷) and pRF4 carrying the dominant allele *rol-6(su1006)* which induces a twist in the body wall⁶. The normal PVM axon clearly extends to the ventral cord, then turns and extends anteriorly along it. The PLM axons extend anteriorly in a subventral position. ALN neuron cell bodies, which also express *mec-7-lacZ*, are indicated with small arrows. The ventral cord is marked by a queue of DAPI-stained neuronal nuclei (arrowheads in b). The animal in c and d is transgenic for evEx27 derived by cotransformation with pNW115 (*mec-7-lacZ*), pPD10.41 (*unc-22* anti-sense which induces a twitching phenotype¹⁵) and pYZ122 (*mec-7-unc-5*). The

errant PVM axon extends dorsally, and when it reaches its dorsal-most extent, turns anteriorly. The PLM neurons are overstained, but at least one PLM axon is visible on the dorsal side. The ventral side is marked by an embryo developing *in utero* (asterisk). The animal in e and f is genetically identical to the animal in a and b. The AVM axon, like the PVM axon in a and b, has a wild-type morphology, that is, it extends to the ventral cord, then anteriorly along the cord into the head. A PLM axon is also visible below the ventral cord in these panels. The AVM axon of the animal in g and h, transgenic for *mec-7-unc-5* (genetically identical to the animal in c and d.) extends to the dorsal side, where it branches anteriorly and posteriorly. h. In the same focal plane as g, shows DAPI-stained nuclei of the multinucleate distal gonad arm (asterisk) marking the dorsal side. The cell body and nucleus of an ALM neuron are also visible in these panels (arrow). Scale bars a-f and g, h, 25 μ m.

post-embryonically, respectively⁹⁻¹¹. We do not know if the displacements observed in *mec-7-unc-5* transgenic animals result from abnormal cell migrations or from unusual tension on the cell bodies produced by the errantly growing axons.

In wild-type animals, UNC-5 is expressed and functions in neurons whose growth cones (GCs) exhibit dorsally oriented pioneering migrations on the epidermis³. Therefore, a logical interpretation of the errant trajectories taken by the touch cell axons in *mec-7-unc-5* transgenic animals is that they result from the normal guidance functions of UNC-5 receptor which resides on the surface of the touch cell GCs. An alternative possibility, however, is that ectopic UNC-5 expression in the touch cells induces dorsally oriented axon trajectories by some mechanism that does not require its normal guidance functions. For example, UNC-5 might act in a dominant negative manner to abrogate the normal guidance mechanisms of the touch cell axons by sequestering a factor required for their guidance. Touch cell axons might then grow dorsally by some unknown

default mechanism. Analysis of double mutants indicates that the guidance functions of *unc-5* require *unc-6* which encodes a secreted, laminin-related protein⁶. Therefore, if the errant touch axon trajectories in *mec-7-unc-5* transgenic animals result from the normal guidance functions of *unc-5*, then these errant trajectories should also require *unc-6*. To test this hypothesis, we passed the synthetic extrachromosomal element carrying *mec-7-unc-5* (plus *mec-7-lacZ* and a cotransformation marker) into a null mutant of *unc-6*. β -galactosidase staining revealed that most touch cell axons in the resultant transgenic *unc-6* mutant animals do not extend dorsally; rather they have trajectories qualitatively indistinguishable from *unc-6* mutant animals that do not carry a *mec-7-unc-5* transgene⁴: most touch cell axons extend forward in a lateral position and many AVM and PVM axons extend ventrally (Fig. 3). Even touch receptor cell bodies appear to be in roughly normal positions in the *unc-6* mutant background (Fig. 3).

It could be argued that the touch cell axons no longer extend

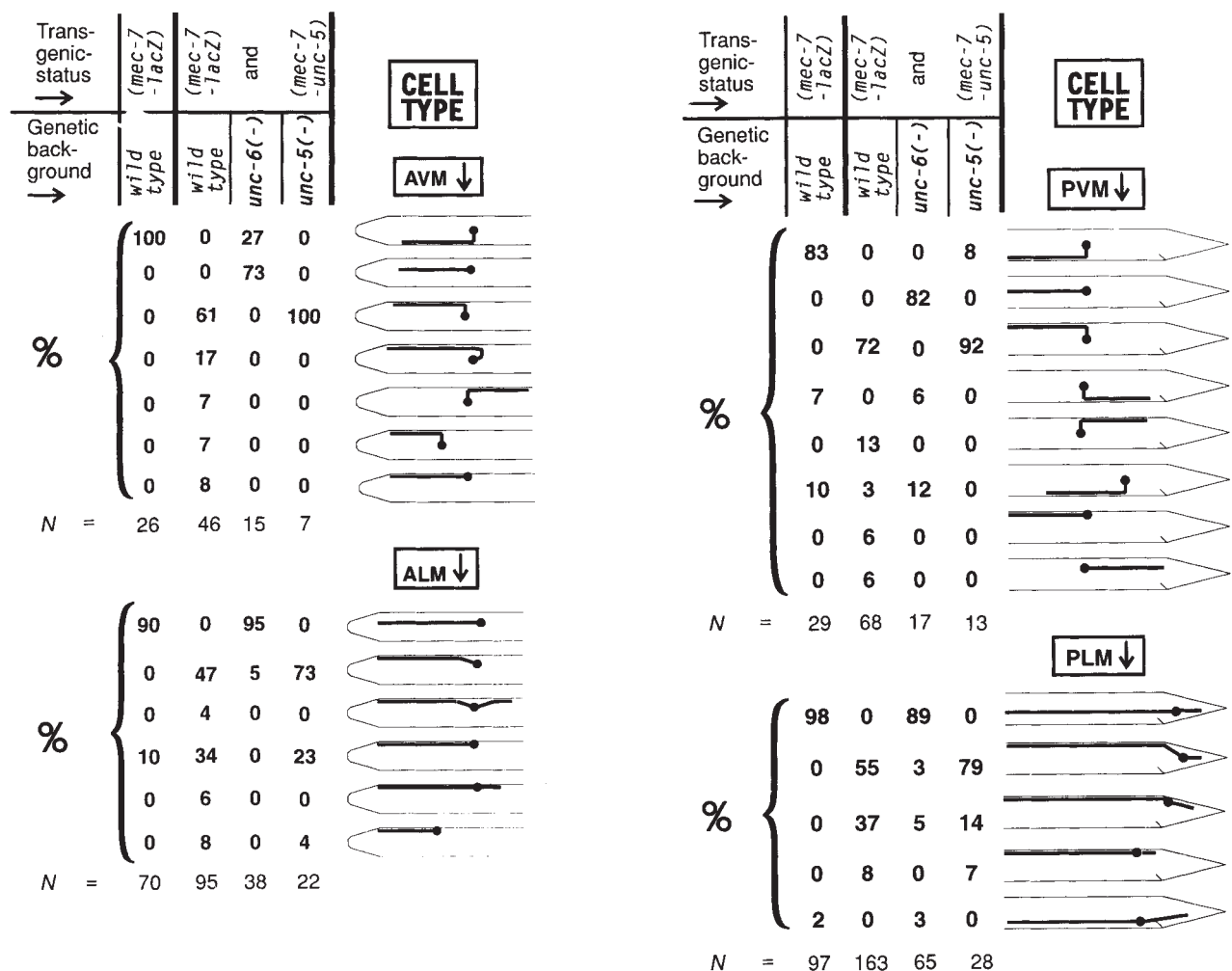


FIG. 3 Summary of touch cell axon trajectories in controls and in animals transgenic for *mec-7-unc-5*. For each cell type, the percentage of cells scored with a given morphology (%) is indicated to the left of each morphological variant. (The topmost cell shown in each group is the morphology most often found in the wild type.) The number of cells observed (*N*) to obtain these percentages is indicated below each cell type group (ALM, AVM, PLM or PVM). The transgenic status and background (chromosomal) genotype of each strain scored is indicated directly above the percentages. Wild type is the Bristol strain N2 (ref. 16). All of the touch cell axons turn along a longitudinal path at the dorsal limit of their trajectory. Most dorsal touch cell axons extend anteriorly, but for AVM (3/46) and PVM (13/68), posterior longitudinal

growth is also occasionally observed. Infrequently (~2 to 5% of cases), touch cell axons in *mec-7-unc-5* transgenic animals have very complex trajectories (not shown), but even in these cases, the axons in the wild type and *unc-5* backgrounds ultimately reach the dorsal side. The touch cell axon trajectories observed in *unc-6* mutant animals transgenic for *mec-7-unc-5* are qualitatively the same as those reported for *unc-6* mutant animals not carrying the transgene⁴. All animals carry extrachromosomal arrays that contain *mec-7-lacZ* and a co-transformation marker (either *rol-6(su1006)* or *unc-22* anti-sense^{9,15}). *mec-7-unc-5* transgenics also carried the *mec-7-unc-5* construct shown in Fig. 1. The transgenic arrays are inherited by only a fraction (~20 to 30%) of self-fertilization progeny, suggesting that they are extrachromosomal.

dorsally in an *unc-6* mutant transgenic for *mec-7-unc-5*, not because guidance on the epidermis is affected, but because *unc-6* animals are depleted of dorsal cord axons. Axons missing from the *unc-6* dorsal cord may be essential for the errant trajectories (induced by the *mec-7-unc-5* transgene) observed in the wild-type genetic background. This possibility was examined by passing the same synthetic *mec-7-unc-5* extrachromosomal element into *unc-5(e53)*, which is also depleted of dorsal cord axons¹². In the resulting transgenic animals, touch cell axons still extend dorsally (69 of 70 axons examined, Fig. 3).

We have shown that UNC-5 expression in the touch neurons suffices to steer their axons in a dorsal direction on the epidermis. This phenomenon is most striking for AVM and PVM axons, which often adopt a trajectory that is the mirror image of their normal one (presumably UNC-5 expression in these cells completely overcomes their normal ventral guidance mechanisms). These results indicate that pioneer GCs that normally migrate dorsally on the epidermis (such as motor neuron GCs), are in most respects equivalent to pioneer GCs that normally migrate ventrally or longitudinally (such as touch neuron GCs). Perhaps the only difference between these GCs is that they have different receptors on their surfaces, which normally respond to different epidermal path cues, but use a similar set of intracellular signalling molecules and/or cytoskeletal associations to do so. An alternative view is that the signalling mechanisms used by motor neuron GCs and touch neuron GCs are different, but that UNC-5 is somehow able to plug into both mechanisms, perhaps using a common adaptor.

It has been previously argued that the *unc-6*-dependent cues that guide dorsally oriented migrations are static, and possibly distributed as a dorsoventral gradient on the epidermis^{4,6}. The fact that the touch neurons have access to these cues is consistent with their possible global distribution throughout the body of the animal.

These results directly demonstrate that *unc-5* and *unc-6* play instructive roles in orienting pioneer axon migrations in a dorsal direction on the epidermis of *C. elegans*. An instructive role for

these genes suggests a simple model for the control of dorsalward turning, which produces the L-shaped migratory trajectory normally exhibited by some migrating motor neuron GCs¹³ and by the migrating distal tip cells of the developing gonad¹⁴. This model presupposes the existence of a simple static pattern of one or a few guidance cues on the epidermis (such as a dorsoventral gradient of an UNC-6 protein). Reorientation from a longitudinal to a dorsally oriented trajectory could then be induced by differentially regulating the expression or function of a single receptor for that cue (such as UNC-5) on the migrating cells. Analogous models have been suggested to explain the reorientation of certain GCs in the vertebrate spinal cord and in the invertebrate central nervous system^{1,2}. An examination of the temporal and spatial expression patterns of UNC-5 in the wild type and in mutants that affect dorsally oriented migrations¹¹ could provide clues to this model for *C. elegans* and possibly for other organisms. □

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Extensive MHC variability in cichlid fishes of Lake Malawi

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LAKE Malawi in East Africa harbours 500–1,000 endemic species of cichlid fishes, all presumably derived by adaptive radiation from a single founding population within the past two million years^{1–3}. The species of this 'flock' differ strikingly in their ecology and behaviour⁴, moderately in their external morphology¹ and very little in their molecular characteristics^{5,6}. Here we describe high sequence variability of class II major histocompatibility complex genes in a sample of species from Lake Malawi. The variability provides a set of molecular markers for studying adaptive radiation and should be useful for estimating the size of the population that founded the species flock.

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Class II major histocompatibility complex (MHC) molecules are membrane-bound heterodimers of α - and β -polypeptides encoded by the *A* and *B* genes, respectively, and are critically involved in the initiation of immune response⁷. In mammals, the functional MHC genes are highly polymorphic, there are often large distances between alleles and the alleles group into lineages which diverged more than 30 Myr ago⁸. If the population that founded the species flock of Lake Malawi was highly polymorphic at the MHC loci and the polymorphism was passed on to the individual species emerging during the adaptive radiation, the extant cichlid species should display large variation at their MHC loci.

To determine the variability of the MHC loci in the cichlids of Lake Malawi, we isolated and sequenced complementary DNA clones from one species (*Aulonocara hansbaenschi*; Fig. 1) and then synthesized oligonucleotide primers corresponding to the ends of exons 1 and 2 of class II *B* genes. We used these primers to amplify, by the polymerase chain reaction (PCR), the entire intron 1 and almost the entire exon 2 from the genomic DNA of members of eight cichlid species of Lake Malawi and four other African cichlid species. From some species we amplified DNA from several individuals, and from each individual one to three amplification products were cloned and sequenced. We obtained 35 intron 1 (Fig. 2) and 37 exon 2 sequences (the amino-acid translation of the latter is given in Fig. 3).

The last 16–18 sites at the 3' end of intron 1 are very similar in all 35 genes (Fig. 2). The rest of the intron seems to be derived from a 12-nucleotide tandem repeat with a consensus sequence ATCAATACACTG. The repeat occurs a different number of

times in different sequences, either contiguously or interrupted by sequences of variable length which seem to be degenerate fragments of the basic 12-mer. According to the total length of intron 1, the number and disposition of the repeats, and the sequence of the interrupting fragments, the 35 sequences can be divided into nine groups, most groups consisting of sequences from different species. The repeats in each group are no more similar to each other than are repeats from different groups.

The amino-acid sequences translated from the exon 2 sequences can also be arranged into groups, each group being characterized by several residues either absent or rare in other groups (Figs 3 and 4). The classification corresponds to that of the intron 1 sequences.

As cichlid fishes are diploid, and as we could obtain three or four different sequences from the same fish (sequences designated by the same number but different letter are from one individual), there must be at least two cichlid class II loci. From experience with mammalian class II sequences, we may assume

that sequences of a single group are alleles. If so, several conclusions can be drawn about the cichlid MHC polymorphism. First, the fact that all the 37 randomly chosen DNA clones differed in their sequences indicates the existence of tremendous MHC variability among the cichlids of the African lakes. Second, the presence of different sequences in different species indicates that extensive MHC polymorphism must have existed in the ancestral population from which the Lake Malawi species flock arose, and that this polymorphism then segregated among the emerging species. Third, the large genetic distances between presumed alleles in the same species (for example, 42 nucleotide substitutions at 290 exon 2 sites compared between *Auha-M-231a* and *Auha-M-231b*) combined with small distances between some of the genes at the same locus in different species (for example, no nucleotide substitution between *Psze-189B* and *Auha-195a*) suggest that MHC polymorphism in cichlids is ancient and that very little new variability has been generated during the period of adaptive radiation in the past two million

Auha-M-231a	-13	CT	TCA	TCC	TTC	CTC	TGC	CTC	AGC	CTC	CTC	TTC	ATC	-1	AGC	CTC	AGC	ACA	GCA	G	Exon 2	AT	GGA	TTT	CTG	AGT	TAT	A	S	AGT	GTG	GAT	CGC	TGT
Auha-M-231b		...	...	...	...	...	...	...	...	...	...	...	...		S	L	S	T	A		D	G	F	L	S	Y		G	V	D	R	C		
Protein		P	S	S	F	L	C	L	S	L	L	F	I		S	L	S	T	A		D	G	F	L	S	Y		G	V	D	R	C		
Auha-M-231a	16	CAG	TTT	AAC	TCC	ACT	GAG	CTG	AAG	GAC	ATC	26	TAC	ATC	AGA	TCT	TCT	GTA	TAT	TAC	AAC	AAG	TTG	GAG	ATT	TTC	AGG	TTC	AGC	AGC				
Auha-M-231b		Q	F	N	S	T	E	L	K	D	I	E	Y	I	TAC	TAC	S	Q	Y	Y	N	K	L	E	I	Y	R	F	S	S				
Protein		Q	F	N	S	T	E	L	K	D	I	E	Y	I	TAC	TAC	S	Q	Y	Y	N	K	L	E	I	Y	R	F	S	S				
Auha-M-231a	44	L	TTG	GGG	AAG	TTT	GTG	GGA	TAC	ACT	GAG	54	TAC	GGA	GTG	AAG	CAG	GCA	GAC	TAC	R	AAC	AA	AT	D	K	AAG	GCA	ATC	CTG	S	S		
Auha-M-231b		S	G	V	G	K	F	V	G	Y	T	E	Y	G	V	K	Q	A	A	G	Y	T	N	D	C	A	CCA	P	A	G	G	CA	Q	
Protein		S	G	V	G	K	F	V	G	Y	T	E	Y	G	V	K	Q	A	A	G	Y	T	N	D	C	A	CCA	P	A	G	G	CA	Q	
Auha-M-231a	72	M	K	GCT	Q	CAG	AAG	GAG	ACG	TAC	TGC	H	N	110	GGT	GTG	D	TAC	AGC	A	I	92	TCT	TCT	AAA	TCA	G	Exon 3	TT	CAA	CCC	TCC		
Auha-M-231b		-R	-G	-G	-G	-G	-G	-G	-G	-G	-G	-A	-C		I	A	C	T	AGC	AA	CC	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG		
Protein		R	R	A	A	K	E	T	Y	C	Q	A	H		I	G	W	Y	S	N	N	L	S	K	S	S	V	Q	P	A	T			
Auha-M-231a	100	GTG	AAG	CTT	CAC	TCC	M	K	AAG	CCT	GCT	TCC	110	AGT	AGT	CAC	R	CAT	CCC	GCC	ATG	TTG	GTC	TGC	AGC	120	GTC	TAT	GAC	TTC	TAT	CCC	AGT	GAG
Auha-M-231b		-G	-G	-G	-G	-G	-G	-G	-G	-G	-G	-G	-G		I	A	C	T	AGC	AA	CC	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	CTG	
Protein		V	K	L	H	S	T	T	P	L	S	S	Q	H	P	A	M	L	V	C	S	V	Y	D	F	Y	P	S	E					
Auha-M-231a	128	ATC	AAA	GTG	AGC	TGG	L	AGA	GAT	GGA	AAG	138	ATA	ACC	TCT	GAT	GTC	ACT	TCC	ACT	GAG	148	GAG	ATG	GCA	GAT	GGT	GAT	TGG	TAC				
Auha-M-231b		-I	-K	-V	-S	-W	-I	-R	-D	-G	-C	-Q	-E	-I	-T	-S	-D	-V	-T	-S	-T	-E	-E	-M	-A	-D	-G	-D	-W	-Y				
Protein		I	K	V	S	W	I	R	D	G	Q	E	I	T	S	D	V	T	S	T	E	E	M	A	D	G	D	W	Y					
Auha-M-231a	156	TAC	CAG	ACC	CAC	TCA	CAC	CTG	GAG	TAC	ACA	166	AGG	TCT	GGA	GAG	AAG	ATC	TCC	TGT	GTG	176	GTG	GAG	CAC	GCC	AGC	TTG	AAG	GAA				
Auha-M-231b		-Y	-Q	-T	-H	-S	-H	-L	-E	-Y	-T	-P	-R	-S	-G	-E	-K	-I	-S	-C	-V	-V	-E	-H	-A	-S	-L	-K	-E					
Protein		Y	Q	T	H	S	H	L	E	Y	T	P	R	S	G	E	K	I	S	C	V	V	E	H	A	S	L	K	E					
Auha-M-231a	184	CCT	CTG	AAA	ACT	GAC	TGG	G	AC	CCG	TCC	ATG	CCT	194	GAG	TCA	GAG	AGG	AAC	AAC	N	V	204	GGA	GCC	TCA	GGA	CTG	ATC	CTG	GGT			
Auha-M-231b		-P	-L	-I	-T	-D	-W	-D	-T	-S	-S	-L	-P	-E	-S	-E	-R	-N	-K	-L	-A	-I	-G	-A	-S	-G	-L	-I	-L	-G				
Protein		P	L	I	T	D	W	D	T	S	S	L	P	E	S	E	R	N	K	L	A	I	G	A	S	G	L	I	L	G				
Auha-M-231a	212	CTG	ATC	TTA	TCT	CTG	GCT	GGA	TTC	ATC	TAC	222	TAC	AAG	AGG	AAG	GCC	CGA	GGT	CGT	ATT	CTG	232	GTT	CCC	AGT	AAC	TGA						
Auha-M-231b		-L	-I	-L	-S	-L	-A	-G	-F	-I	-Y	-Y	-K	-R	-K	-A	-R	-G	-R	-I	-L	-V	-P	-S	-N									
Protein		L	I	L	S	L	A	G	F	I	Y	Y	K	R	K	A	R	G	R	I	L	V	P	S	N									
Auha-M-231a	3'UT region	1	GACTGTTTCT	11	GGTCCCTGGTC	21	CTGTCTGATG	31	TTATAAAGC	41	AGCTGCTTCA	51	TGTGTTTCA	61	AGACTGATGA	71	TGATGATGAT	81	GATCTCTGTC	91	TGGACTCTGA													
Auha-M-231b		...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Auha-M-231a	101	ACCTGATCCA	111	GGATCTCAAT	121	GTGTTGGTGG	131	GTTGTTGTGG	141	TTTCAC*TTG	151	GATGATCCTG	161	GATCAGGTCT	171	AATGTCGACT	181	CTGCTGTTTG	191	ATGCTTCACT														
Auha-M-231b		...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Auha-M-231a	201	GTTTAACTCT	211	GCTCTTCAAT	221	CCATGTAATA	231	TAATCCCTGT	241	AATCCAGGTC	251	TCAGGGTGAC	261	TTTGTCTCT	271	CTGCTCATCT	281	GATTCTAATT	291	TACAGATTTT														
Auha-M-231b		...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Auha-M-231a	301	AACTTTTCATG	311	TCAAAATAAA	321	AT.....	331																											
Auha-M-231b		...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	

FIG. 1 Nucleotide sequence of MHC class II B cDNA clones *Auha-M-231a* and *Auha-M-231b* obtained from the cichlid fish *A. hansbaenschi*. (The MHC sequences of the different fish are designated by an amalgamation of the first two letters of the genus and the first two letters of the species scientific names, followed by the one-letter code for the lake or river of origin, the serial number of the individual, and the serial number of the sequence.) Dashes indicate identity with the top sequence, dots unavailability of sequence information. Translation into amino-acid sequence appears under the *Auha-M-231b* nucleotide sequence and is given in the single-letter code. Differences in protein sequence between *Auha-M-231a* and *-231b* are indicated above the *231a* nucleotide sequence. Leader sequence is numbered from -1 to -13, the mature protein sequence from 1 to 235; the 3'-untranslated region nucleotide sequence is numbered separately. Vertical lines indicate exon boundaries inferred from both genomic sequence and comparison with mammalian genes.

METHODS. The messenger RNA was isolated from spleen and hepatopancreas¹¹. Poly(A)⁺RNA was separated using the Pharmacia LKB kit. The cDNA was synthesized and *EcoRI* adaptors were added using the Pharmacia LKB cDNA synthesis kit. The cDNA library was constructed in the λ gt10 vector (Stratagene), the ligated cDNA was packaged into λ phages using the Stratagene packaging kit, and the phages were used to infect *Escherichia coli* C600 hfl bacteria. The complexity of the library was 8×10^5 plaque-forming units (PFU). The library was amplified once to 1.5×10^{10} PFU. For screening the library, replicate nitrocellulose filters (Sartorius) containing 5×10^5 PFU were hybridized with the zebrafish probe 5E1 which encompasses exon 3 of a class II B gene⁹. The probe was labelled by the random priming method to a specific activity of 1×10^9 c.p.m. μg^{-1} . Six of the nine positive clones were positive on rescreening. Of these, two clones were isolated, subcloned in the Bluescript II vector and sequenced by the dideoxy chain-termination method¹².

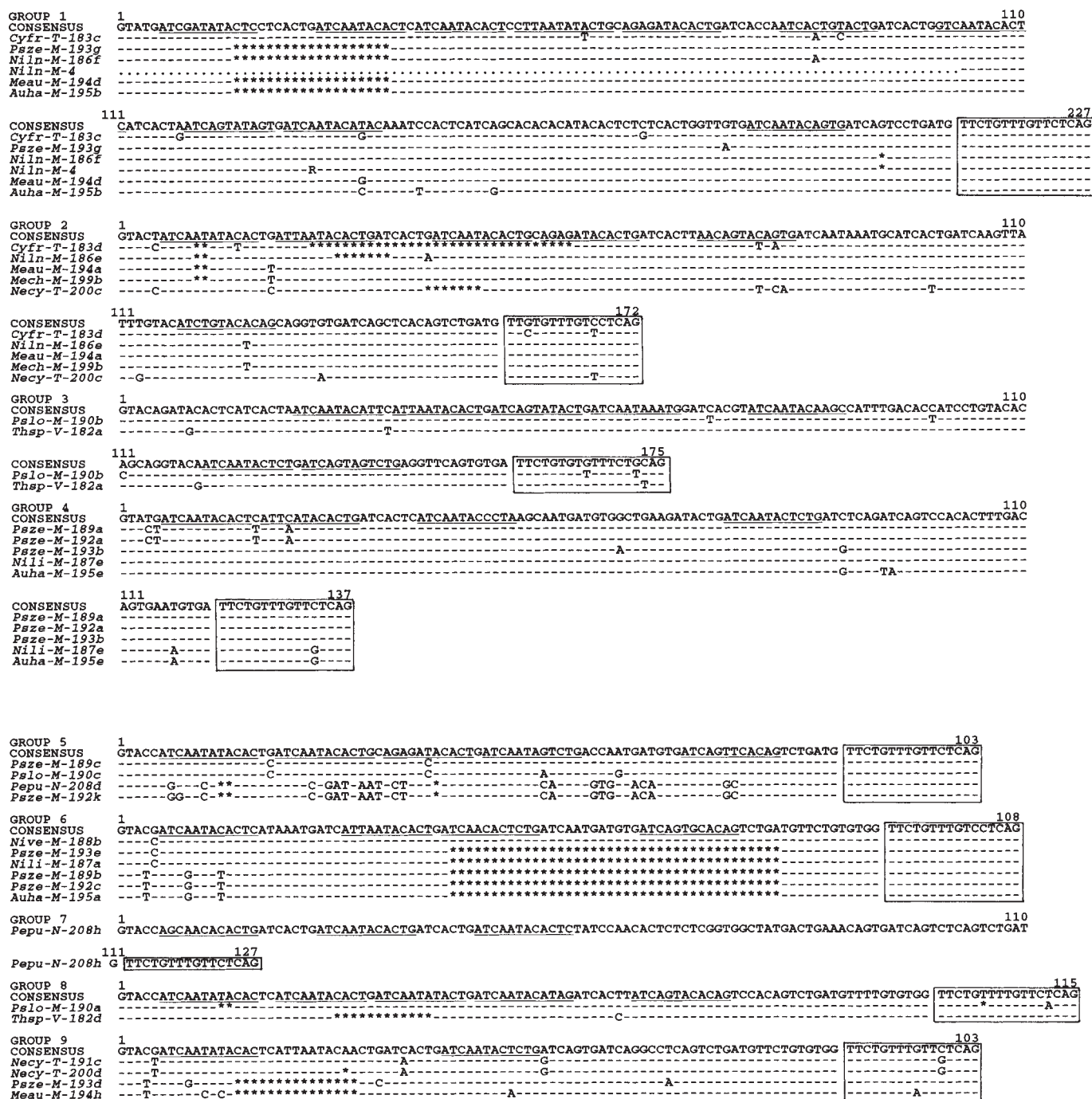


FIG. 2 Intron 1 sequences from MHC class II B genes of cichlid fish. Identity with the simple majority consensus sequence is indicated by dashes, unavailability of sequence information by dots and postulated deletions (insertions) by asterisks. The sequences are arranged in groups according to their length and overall similarity. The conserved 3' flank is boxed, the repeats are underlined. The sequences were obtained from the following fish: *Thsp*, *Thoracochromis spec.*; *Auha*, *A. hansbaenschi*; *Cyfr*, *Cyphotilapia frontosa*; *Nili*, *Nimbochromis livingstonii*; *Nili*, *Nimbochromis nilii*; *Nive*, *Nimbochromis venustus*; *Meau*, *M. auratus*; *Mech*, *Melanochromis chipokae*; *Pslo*, *Pseudotropheus lombardoi*; *Nely*, *Neolamprologus cylindricus*; *Pepu*, *Pelvicachromis pulcher*; *Psze*, *P. zebra* complex. Origin of fishes: M, Lake Malawi; N, Niger River; T, Lake Tanganyika; V, Lake Victoria.

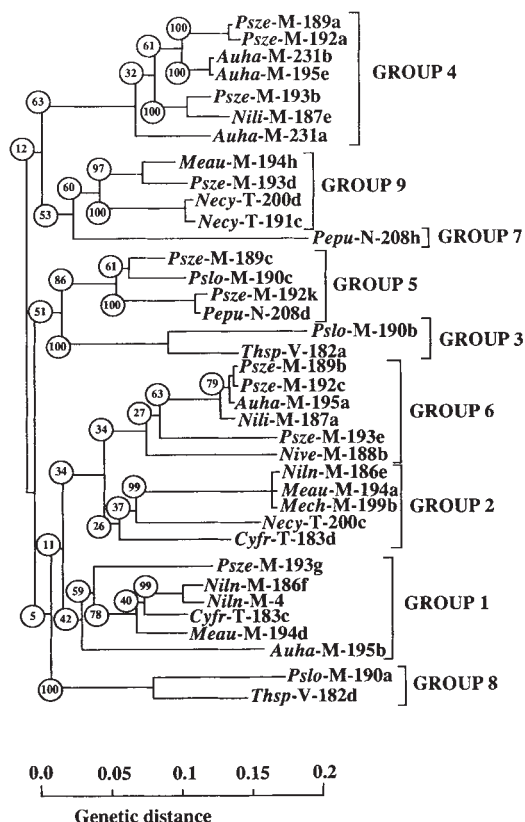
METHODS. The sequences were obtained by the dideoxy chain-

termination method¹² from genomic DNA amplified with 1 unit *AmpliTag* polymerase per 50 µl of reaction volume, 5 µl of 10 × PCR buffer, 0.5 µM of each primer, and 0.2 mM of each deoxynucleotide. The conditions of the PCR were: denaturation for 2 min at 94°C followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 55°C and primer extension for 2 min at 72°C; the final extension was for 10 min at 72°C. The sequences of the primers were: Tu383 (3' end of exon 1): 5'-CTCTTCATCAGCCTCAGCACA-3'; Tu377 (degenerate primer, 3' end of exon 2): 5'-TGATTAGACAGA (G/A)(T/G)(T/G)(T/C)GCTGTA-3'; Tu422 (3' end of exon 2, based on the *Auha*-M-231a sequence): 5'-TGATTAGACAGAATGGCGCTGTA-3'; Tu423 (3' end of exon 2, based on the *Auha*-M-231b sequence): 5'-TGATTAGACAGA-GGGTGTGCTGTA-3'. The primers were used in the combination Tu383-Tu377 or Tu383-Tu422+Tu423.

	5	15	25	35	45	55	65	75	85
CONSENSUS ==>	GFMVMVNR	CDFNSTELKD	IEYILSYYYN	KIEYTRFDSS	VGKPFVGYTEY	GVKNAEYWNK	DPGQLAAWRA	QKETVCQHNI	GVW
1	Cyfr-T-183c	-----S-----	-TF-S-----	MM-L-----	-E-----KL	-----DRV--	-Q-----V--	-----G-----L	--Y
	Psze-M-193g	-----S-I-----	-----Q-----	TM-IL-----	-EY-----QL	-----DR--	-----V--VR--	-----G-----V	--D
	Niln-M-186f	-----A-----	-QF-M-----	MM-IL-----	-EY-----QL	-N-DRL--	-----R--	-----G-----V	--D
	Niln-M-4	-----A-----	-QF-M-----	MM-IL-----	-EY-----QL	-N-DRL--	-----G-R--	-----G-----V	--D
	Meau-M-194d	-----S-G-----	-DP-----	MM-I-----	-----F-QL	-----DRL--	-Q-----	-----G-----V	--D
	Auha-M-195b	-----N-RMA--	-V-----	-QF-D-----	MM-VV-----	-EY-----DF	-Q-K-F-S	I-----SE--	-----HN--LD
	Cyfr-T-183d	-----K-----TQ	-----K-V-----	-----VN--D	-HY-----	-----L--N	-QA-M-V--	-----G-----L	--Y
2	Niln-M-186e	-----K-----T-D	-N-----	-Q-V-----	-----VN--D	-----K-L--*	SGAY-TQVKT	E-G-----L	-----
	Meau-M-194a	-----K-----T-D	-N-----	-Q-V-----	-----VN--D	-----L--*	SGAY-TQVKT	E-G-----L	-----
	Mech-M-199b	-----K-----T-D	-N-----	-Q-V-----	-----VN--D	-----L--*	SGAY-TQVKT	E-G-----L	-----
	Necy-T-200c	-----K-----M-----	-----QF-S-----	-----VN--D	-----	-----T-----N	-AI-TQVK	E-----V--V	-LD
3	Pslo-M-190b	-----LE-N-W--	-V-T-PD--	-----FYF-N--	-----KSIE-S-	L-----N-V	-Q-A-----	-AE--VR--	E--R--VP--ID
	Thsp-V-182a	-----LE-RMD--	-V-T-PD--	-----YS-N--	-----K-VE-S-	L-Y-----N-V	-Q-A-----	-Q-M--LK--	R-----L--I-
	Psze-M-189a	-----LS-S-E--	-Q-----	-----L-Q-----	-L-IY-S-	L-----	-Q-K-F-D	L--EV-R-T-	E--R-----N-
	Psze-M-192a	-----LS-S-E--	-Q-----	-----L-Q-----	-L-IY-S-	L-----	-Q-K-FSD	L--EV-R-T-	E--R-----N-
	Psze-M-193b	-----LS-R-S--	-Q-----	-----Y-Q-----	-L-IY-S-	L-----	-Q-K-F--	TAYVSSLN-	-----V--I-
4	Niln-M-187e	-----YLD-Y--	-Q-----	-----Y-A-----	-L-IY-S-	L-----	-Q-K-F--	TAYVSSMK-	-----V--I-
	Auha-M-195e	-----LS-G-D--	-Q-----	-----Y-Q-----	-L-IY-S-	L-----	-Q-K-F-D	Q-AVV-QR--	A-----I-
	Auha-M-231b	-----LS-G-D--	-Q-----	-----Y-Q-----	-L-IY-S-	L-----	-Q-K-F-D	Q-AVV-QR--	A-----I-
	Auha-M-231a	-----LS-S-A--	-Q-----	-----R-V-----	-L-IF-S-	L-----	-Q-D-R-N	KAI-SSMK-	-----HN--D
	Psze-M-189c	-----LE-S-D--	-----	-----F-I--F-	-V-DV-S-	-----P	-L-Y-AD--	-Q-D--M--	-----L--D
5	Pslo-M-190c	-----PPE-Q-A--	-V-----	-----I--F-	-V-DV-S-	-----Y--P	-L-Y-AD--	-Q--SE--	-----L--D
	Pepu-N-208d	-----LE-A-I--	-V-----	-----F-R--F-	-V-DV-S-	-----Y--P	-L-Y--N--	QALM-SE--	-----DID
	Psze-M-192k	-----LE-A-I--	-V-----G	-----F-R--F-	-V-DV-S-	-----Y--P	-L-Y--N--	QALM-SEK-	-----DID
	Nive-M-188b	-----E--TAT--	-N-----	-Q-S-----FR	-V-F-----	-----Y--	-----N	Q--SE--	-----V--V--N-
	Psze-M-193e	-----K--MD--	-N-----PQ	-----R-----	-----V-----D	-----HY--	-----T--N	-AM--D--	A--R--V--N-
6	Niln-M-187a	-----K--W--	-N-----S	-----V--F--	-----	-----HY--	-----N	L--R--	A-----
	Psze-M-189b	-----K--W--	-N-----	-----V--F--	-----	-----HY--	-----N	L--V--R--	A-----N-
	Psze-M-192c	-----K--W--	-N-----	-----V--F--	-----	-----HY--	-----N	L--V--R--	A-----N-
	Auha-M-195a	-----K--W--	-N-----	-----V--F--	-----	-----HY--	-----N	L--E--R--	A-----N-
7	Pepu-N-208h	-----S-T-V--	-S-----	-----F-Y-H-H	-----M--S-	-----R--F--	-EF--DNL-	NQEVMSMK-	-----R--N-
8	Pslo-M-190a	-----Y-DL--	-V-----V	-----VR-W-C	-L-II--	-----L	-----A--	-Q--TF-S	L--V--K--N-
	Thsp-V-182d	-----Y-NLA--	-V-----	T-F-V-W-C	-L-FI--	-----L	-----KG-A--*	KQ-D--V--	-----T--N-
9	Necy-T-191c	-----E-H-W--	-----	-----F-Y-I--	-----I--S-	-----F-K--	-N-DRV--	-Q--V--	-----QH--
	Necy-T-200d	-----E-Y-W--	-----	-----F-Y-M--	-----I--S-	-----F-K--	-N-DRV--	-Q--V--	-----QH--
	Psze-M-193d	-----E-Y-W--	-----	-----TA-----	-----A--S-	-----EY--F-K--	-H-DI--	-QA-M--	-----LP--ID
	Meau-M-194h	-----E-C-W--	-----	-----FT-----	-----A--S-	-----EY--F-KF	-H-DI--	-Q-D--M--	-----E

FIG. 3 Amino-acid sequence of cichlid class II MHC β -chains. The sequence was translated from exon 2 nucleotide sequences that have been deposited at GenBank. Identity with the simple majority consensus sequence is indicated by a dash, a gap by an asterisk. The

sequence is given in the standard single-letter code. The designations of the individual sequences and the groups (numbering far left) are explained in Fig. 2. The numbering follows that of the zebrafish⁹.



years. Fourth, the similar MHC polymorphism found in lacustrine and riverine species (for example, *Psze-192k* from Lake Malawi and *Pepu-208d* from the Niger River differ by two substitutions only) suggests that it will be possible to trace the genes of the extant species in the African lakes back to ancestors in the African rivers. Fifth, a variability plot (data not shown) indicates that nonsynonymous substitutions are concentrated in certain regions of exon 2 which correspond to the highly variable regions in functional mammalian MHC genes. This observation, which confirms similar data obtained for zebrafish⁹, suggests that the fish MHC genes have a similar function to their mammalian counterparts — they specifically distinguish self from nonself (parasite-derived) peptides.

Overall, our data suggest that the population that founded the species flock of Lake Malawi was highly polymorphic at its MHC loci. In a parallel study¹⁰, we demonstrate that two Lake Malawi species (*Pseudotropheus zebra* and *Melanochromis auratus*) have nonoverlapping profiles of class II *B* genes. Hence, during radiation different MHC alleles apparently distributed (fortuitously or selectively) into different species. Further studies should therefore allow the tracing of phylogenetic lineages through their MHC polymorphism and estimation of the size of the founding population, and should also help elucidate the manner of speciation during adaptive radiation. The high MHC

FIG. 4 Dendrogram of the cichlid class II MHC exon 2 sequences constructed by the neighbour-joining method¹³. Genetic distances were estimated using Kimura's two-parameter method¹⁴. Numbering on nodes indicates the number of times a particular branch is recovered per 100 bootstrap replications following 500 replications.

variability demonstrated here should also provide a molecular tool for a variety of ecological and ethological studies of the cichlid fishes. □

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Genes required for GABA function in *Caenorhabditis elegans*

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γ -AMINOBUTYRIC ACID (GABA) neurotransmission is widespread in vertebrate and invertebrate nervous systems¹. Here we use a genetic approach to identify molecules specific to GABA function. On the basis of the known *in vivo* roles of GABAergic neurons in controlling behaviour of the nematode *Caenorhabditis elegans*², we identified mutants defective in GABA-mediated behaviours. Five genes are necessary either for GABAergic neuronal differentiation or for pre- or postsynaptic GABAergic function. The gene *unc-30* is required for the differentiation of a specific type of GABAergic neuron, the type-D inhibitory motor neuron. The gene *unc-25* is necessary for GABA expression and probably encodes the GABA biosynthetic enzyme glutamic acid decarboxylase. The genes *unc-46* and *unc-47* seem to be required for normal GABA release. Finally, the gene *unc-49* is apparently necessary postsynaptically for the inhibitory effect of GABA on the body muscles and might encode a protein needed for the function of a GABA_A-like receptor. Some of these genes are likely to encode previously unidentified proteins required for GABA function.

Different classes of GABAergic neurons of the nematode *Caenorhabditis elegans* are required for different behaviours². The single AVL and DVB neurons stimulate the enteric muscles during the defaecation cycle, the four RME motor neurons modulate the head movements necessary for foraging behaviour, and the six DD and 13 VD cells are inhibitory motor neurons

required for locomotion. Animals in which the DD and VD neurons have been killed with a laser microbeam simultaneously contract antagonistic dorsal and ventral body muscles², causing these animals to shrink. This understanding of the *in vivo* functions of the *C. elegans* GABAergic neurons allowed us to identify and analyse mutant strains with abnormal GABA function.

Mutations in five genes, *unc-25*, *unc-30*, *unc-46*, *unc-47* and *unc-49* (ref. 3), cause animals to shrink⁴. Because the animals shrink, they behave as if they lack DD and VD function. By examining other behaviours of these mutants, we determined whether the GABA functions of AVL, DVB and the RMEs are also defective. We also examined GABA expression and GABAergic nerve process morphology by staining worms with an anti-GABA antibody (Fig. 1). Finally, we assayed GABA receptor function by measuring the sensitivities of the mutants to the GABA agonist muscimol: when wild-type worms are treated with muscimol, muscle contractions cease in all muscles and the worms develop a flaccid paralysis (Fig. 2a; and M. Chalfie, personal communication). Muscimol sensitivity seems to be mediated at the muscle membrane⁵, so that resistance to muscimol indicates a defect in the postsynaptic response to GABA. These five genes can be divided into three classes based on the aspect of GABA neurotransmission for which they are required: neuronal differentiation, presynaptic function, and postsynaptic function.

Differentiation. The gene *unc-30* is required for the differentiation of the two anatomically related⁶ classes of type-D GABAergic neurons, the DD (dorsal D) and VD (ventral D) ventral cord motor neurons. *unc-30* mutants display the shrinking locomotory defect of animals in which the DD and VD neurons have been killed by a laser microbeam², but not the foraging defect of animals that lack the RME neurons or the defaecation cycle defects of animals that lack the AVL and DVB neurons (Table 1). Antibodies against GABA failed to stain the DD or VD motor neurons in *unc-30* mutants but did stain all other GABAergic neurons normally (Fig. 1b), again indicating that of the 26 GABAergic neurons, only the VD and DD neurons are abnormal. Reconstructions of the VD and DD neurons of *unc-30* animals from electron micrographs of serial sections revealed that the VD and DD neurons form synapses with incorrect targets, despite being grossly normal in pathfinding (J. G. White, E. Southgate, J. N. Thomson and S. Brenner, manuscript in preparation). *unc-30* animals appeared to be normal in postsynaptic GABA function: *unc-30* animals responded to muscimol like wild-type animals (Fig. 2a). These observations indicate that *unc-30* is required for the differentiation of the DD and VD classes of GABAergic neurons.

Presynaptic function. The gene *unc-25* is necessary for the expression of GABA in all GABAergic neurons. Our behavioural studies indicated that *unc-25* animals have defects in all of the functions mediated by GABAergic neurons (Table 1). No additional behavioural defects were observed in these mutants, indicating that there are no gross abnormalities in the non-GABAergic nervous system. GABA expression is eliminated in *unc-25* mutants as assayed by immunocytochemistry (Fig. 1c). Postsynaptic GABA function apparently remains intact in *unc-25* mutants, since they were sensitive to the GABA agonist muscimol (Fig. 2a). These results suggest that it is the reduced level of GABA in *unc-25* animals that causes the behavioural defects observed, but these results do not exclude the possibility that *unc-25* mutations cause defects in other aspects of GABAergic function in addition to GABA expression.

To demonstrate that other presynaptic GABAergic functions are intact in *unc-25* mutants, we showed that restoring GABA levels in the AVL and DVB neurons was sufficient to restore function to these neurons. When *unc-25* animals were bathed in GABA, the AVL and DVB neurons accumulated GABA as detected by immunocytochemistry (Fig. 1d), and the enteric muscle contractions mediated by AVL and DVB were restored

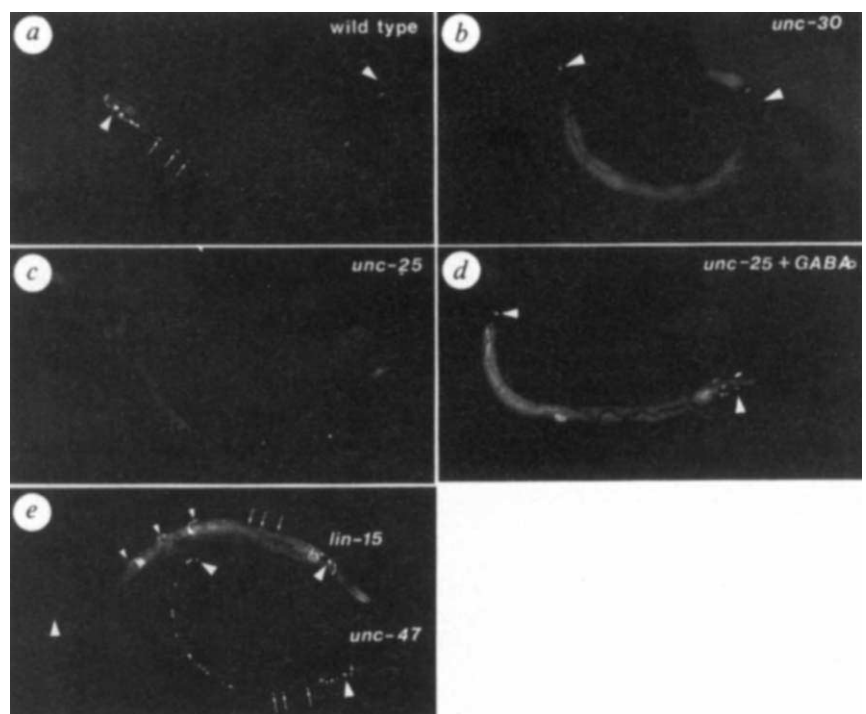
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TABLE 1 Mutant phenotypes

Genotype	Behaviour			GABA expression				Muscimol sensitivity	Proposed site	Proposed defect
	Locomotion (DDs and VDs)	Defecation (AVL and DVB)	Foraging (RMEs)	DDs and VDs	AVL and DVB	RMEs	RIS			
Wild type	+	+	+	++	++	+	+	+		
<i>unc-30</i>	Shrinking	Expulsion defective	+	++	++	+	+	+	Presynaptic	DD, VD differentiation
<i>unc-43</i>	Shrinking	Expulsion defective	+	++	None	None	None	+	Presynaptic	GABA synthesis
<i>unc-44</i>	Shrinking	Expulsion defective	+	++	++	++	++	+	Presynaptic	GABA release
<i>unc-46</i>	Shrinking	Weak expulsion	Weak loopy	++	++	+	+	+	Presynaptic	GABA release
<i>unc-49</i>	Shrinking	Defective	+	++	++	+	+	Resistant	Postsynaptic	GABA _A receptor

One plus sign means, like wild type; for GABA expression, two plus signs indicate abnormally high intensity of staining. Locomotion was assayed by touching worms on the nose with a platinum wire while observing them with a dissecting microscope. An animal was scored as a shrinker if it failed to move backwards and it decreased in body length. Defaecation was assayed by observing with a dissecting microscope whether an enteric muscle contraction occurred following a posterior body contraction (PBOC) during the defaecation cycle¹³. Animals scored as defective displayed the expulsion-defective phenotype¹³. Foraging was assayed in a double-blind test. About ten animals of each genotype were placed on agar plates with no bacteria, plates were coded and mixed and foraging was assayed. Foraging was scored as loopy if animals displayed exaggerated bends of the head while moving forward². Data for enteric muscle contractions per posterior body contraction and for foraging behaviour were as follows: wild type: 101/110 = 0.92, non-loopy; *unc-30*(e191) 85/88 = 0.97, non-loopy; *unc-30*(e165) 54/55 = 0.98, non-loopy; *unc-25*(e156) 9/76 = 0.12, loopy; *unc-25*(e891) 11/55 = 0.20, loopy; *unc-25*(e265) 8/55 = 0.15, loopy; *unc-47*(e307) 17/110 = 0.15, loopy; *unc-47*(e542) 16/88 = 0.18, loopy; *unc-47*(n2252) 21/165 = 0.13, loopy; *unc-46*(e177) 33/88 = 0.38, weak loopy; *unc-46*(e300) 32/110 = 0.29, weak loopy; *unc-46*(e642) 62/110 = 0.56, weak loopy; *unc-49*(e382) 54/55 = 0.98, non-loopy; *unc-49*(e407) 87/88 = 0.99, non-loopy; *unc-49*(e641) 85/88 = 0.97, non-loopy. GABA expression was assayed by anti-GABA staining. Muscimol sensitivity was assayed quantitatively based upon its effects on pharyngeal pumping (Fig. 2) or qualitatively based upon body muscle paralysis in the same experiments. All *unc-49* alleles tested conferred resistance to muscimol: *unc-49*(e382), *unc-49*(e407), *unc-49*(e468), *unc-49*(e641), and *unc-49*(e929). Additional screens for shrinker mutants have identified only mutations in the existing shrinker genes (Y. Jin, J. Kaplan, E.J. S.M. and H.R.H., unpublished observations). Of the six genes previously reported to be able to mutate to produce a shrinker phenotype⁴, one (*unc-43*) appears to be different: *unc-43* mutants have a distinctive locomotory defect and do not have obvious abnormalities in foraging or defaecation (data not shown). For this reason, we have not included *unc-43* among the genes described here.

FIG. 1 GABA immunoreactivity in wild-type and mutant *C. elegans*. In all photographs, the ventral cord neurons DD2, VD3 and VD4 (arrows) and the head neuron AVL and the tail neuron DVB (large arrowheads) are indicated when they stained. **a**, Wild type. Twenty-six cells stain: 6 DDs, 13 VD3s, 4 RMEs, AVL, DVB and RIS². **b**, *unc-30*(e191). The DD and VD ventral cord motor neurons did not stain. These cells were still present, as they could be visualized with the nuclear stain 4-6-diamidino-2-phenylindole dihydrochloride (DAPI)¹⁷ (data not shown). The defect in GABA expression in *unc-30* animals is not a consequence of their minor abnormalities in pathfinding, because studies of other mutants have shown that abnormal pathfinding itself does not affect GABA expression in the DD and VD neurons¹⁷. **c**, *unc-25*(e156). No cells stained, although they are generated and occupy their normal positions, as assessed by staining cell nuclei with DAPI (data not shown). **d**, *unc-25*(e156)+GABA. *unc-25*(e156) animals grown overnight in Petri dishes on agar seeded with *Escherichia coli*³ and containing 100 mM GABA were stained for GABA. In addition to the neurons AVL, DVB and RIS, several normally non-GABA-staining neurons in ganglia of the head and tail accumulated GABA after this treatment. The identities of these cells have not yet been determined. These extra cells also accumulated GABA when wild-type animals were treated with GABA (data not shown). The RMEs stained variably. **e**, *unc-47*(e307) and *lin-15*(n765). To provide an internal control, *lin-15* animals were fixed and stained in the same test tube as the *unc-47* animals. *lin-15* animals can be distinguished by their multiple pseudovulval protrusions¹⁸ (small arrowheads). GABAergic neurons in *unc-47* animals reproducibly stained more intensely than neurons in wild-type (data not shown) or *lin-15* animals. We stained mutants carrying other alleles of these genes and observed similar staining patterns: *unc-30* (e165, e191, e318, e595, e646, e727, e926, and e1169); *unc-25* (e156, e265, e591,



e891 and sa4); and *unc-47* (e307, e542, and e707). *unc-46*(e177, e300 and e642) and *unc-49*(e382, e407, e468, e641 and e929) animals stained the same as wild-type animals (data not shown). **METHODS.** Animals were fixed, permeabilized and stained with anti-GABA antisera as previously described¹⁷. *unc-47* animals consistently stained more intensely than other animals of various genotypes in blind assays of preparations of single strains or mixtures of test animals and morphologically distinguishable control animals stained in the same test tube.

TABLE 2 Effect of exogenous GABA and nipecotic acid on *unc-25* mutants

Genotype and cells killed	EMC/def. cycle	None			30 mM GABA			30 mM GABA 10 mM nipecotic acid			10 mM nipecotic acid		
		No. def. cycles	No. animals		EMC/def. cycle	No. def. cycles	No. animals	EMC/def. cycle	No. def. cycles	No. animals	EMC/def. cycle	No. def. cycles	No. animals
Wild type	0.97	33	3		0.84	33	3	1.00	66	6	0.97	33	3
<i>unc-25</i>	0.12	77	7		0.87	175	14	0.13	143	13	0.17	99	9
<i>unc-25</i> AVL ⁺ DVB ⁺	0.01 + 0.02*	118	7		0.00	96	6	—	—	—	—	—	—

Enteric muscle contractions (EMC) were scored for each defaecation cycle (def. cycle). Note that nipecotic acid blocked the restoration of enteric muscle contractions to *unc-25* mutants by GABA but did not act either as an antagonist for GABA (nipecotic acid did not block enteric muscle contraction in the wild type) or as an agonist (nipecotic acid did not restore enteric muscle contraction to *unc-25* mutants on its own). AVL⁺ DVB⁺, the neurons AVL and DVB were killed with a laser. EMC/def. cycle, ratio between the number of enteric muscle contractions and the number of defaecation cycles observed; no def. cycles, number of defaecation cycles observed; no animals, number of animals observed. Asterisk, the value following the plus sign represents enteric muscle contractions that occurred between defaecation cycles, that is, more than 10 s following one posterior body contraction and preceding the next. Young adults (about 20 h after the L4 moult) were placed on agar seeded with bacteria, and the defaecation cycle¹³ was observed using a dissecting microscope. Animals were placed on agar containing GABA and nipecotic acid for 3 h before observation to allow penetration of the drug. Treated animals were stained after the assay: rescued animals stained and nipecotic acid-blocked animals failed to stain with the anti-GABA antisera. Laser-operated animals were anaesthetized on 5% agarose pads containing 10 mM sodium azide¹⁵ and mounted under a coverslip. Cells were killed using a laser focused through a microscope^{7,14}. Wild-type control animals were anaesthetized in parallel with the experimental animals but had no cells killed. The RMEs took up GABA variably, and normal foraging could be restored in *unc-25* animals by exogenous GABA (data not shown). Interestingly, the DD and VD neurons did not take up GABA in *unc-25* animals (Fig. 1d), and locomotion was not restored. This observation suggests that these neurons do not express the GABA membrane transporter. Supporting this hypothesis, the equivalent GABAergic neurons of the larger nematode *Ascaris suum* fail to take up radiolabelled GABA in cut animals¹⁵.

(Table 2, row 2). Did GABA rescue AVL and DVB function by a presynaptic or a postsynaptic mechanism? To answer this question, we determined whether rescue required the presence of AVL and DVB and of a functional system of GABA reuptake. Using a laser microbeam⁷, we killed AVL and DVB in *unc-25* animals and observed that exogenous GABA no longer restored enteric muscle contractions (Table 2, row 3). We also found that nipecotic acid, which specifically blocks the plasma membrane GABA transporter in invertebrates as well as vertebrates¹, prevented the accumulation of GABA in AVL and DVB (data not shown) as well as the restoration of AVL and DVB function (Table 2). (Nipecotic acid had no effect on the behaviour of wild-type animals.) These observations indicate that if *unc-25* animals are provided with exogenous GABA, AVL and DVB are capable of normal GABA uptake, release and stimulation of the enteric muscles.

Thus, the principal defect in *unc-25* mutants appears to be a simple lack of GABA. GABA is synthesized from glutamate in a single step by the enzyme glutamic acid decarboxylase (GAD) in vertebrates¹ and in *C. elegans*⁸. It seems likely that the *unc-25* gene encodes GAD. Consistent with this hypothesis, GAD activity is greatly reduced in *unc-25* mutant extracts (C. Johnson, personal communication), and a *C. elegans* GAD gene identified on the basis of its sequence similarity to a mammalian GAD gene⁹ maps near the *unc-25* gene (A. Coulson, personal communication).

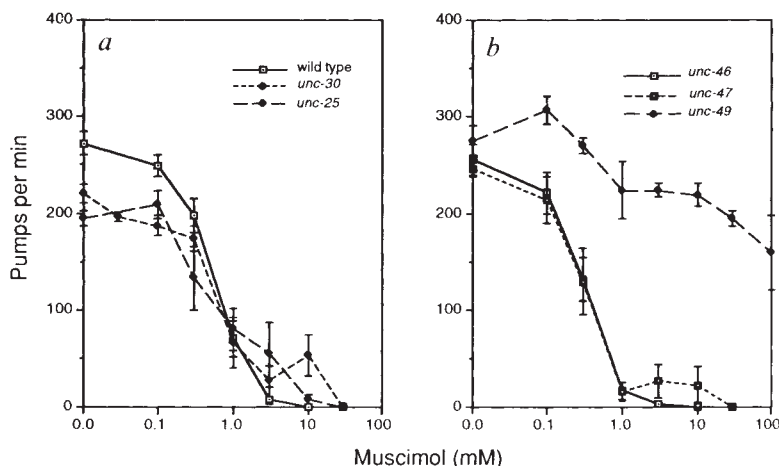
The genes *unc-46* and *unc-47* seem to be necessary for the normal release of GABA into the synaptic cleft. Like *unc-25* mutants, *unc-46* and *unc-47* mutants displayed specific defects in all behaviours mediated by GABAergic neurons (Table 1),

although these defects were weaker in *unc-46* animals. *unc-47* animals have an unambiguous presynaptic defect: immunocytochemical studies revealed that all GABAergic neurons in *unc-47* animals contained abnormally high levels of GABA (Fig. 1e). GABA expression appeared roughly normal in *unc-46* animals (data not shown), although only severe changes in GABA expression would be detectable by immunocytochemistry. Because both *unc-47* and *unc-46* mutants displayed normal sensitivity to muscimol (Fig. 2b), it seems likely that postsynaptic GABA functions are intact in *unc-46* and *unc-47* mutants and that the defects in these mutants are presynaptic. We postulate that mutations in the *unc-47* and *unc-46* genes cause a defect within the GABAergic neurons themselves, such as in the vesicular transporter necessary for uptake of GABA into synaptic vesicles¹⁰ or in a biochemical function required for normal GABA release.

Postsynaptic function. The gene *unc-49* appears to be required for the inhibitory effect of GABA on the body muscles but not for the excitatory effect of GABA on the enteric muscles. First, *unc-49* mutants displayed the locomotory (shrinker) defect seen in animals with the DD and VD motor neurons killed but had normal contractions of the enteric muscles (Table 1). *unc-49* mutants were also normal in foraging. Second, GABA expression was normal in *unc-49* animals (data not shown). Interestingly, *unc-49* animals were resistant to the paralyzing effects of muscimol (Fig. 2b). This observation indicates that the inhibitory GABA receptor responsible for mediating the effects of muscimol is disrupted. The *unc-49* gene might encode a subunit of an inhibitory receptor such as a GABA_A-like receptor¹ or another protein necessary for receptor function.

FIG. 2 Effect of muscimol on pharyngeal pumping. a, Pharyngeal pumping in wild-type, *unc-30(e191)* and *unc-25(e156)* animals. b, Pharyngeal pumping in *unc-46(e177)*, *unc-47(e307)*, and *unc-49(e382)* animals. Strains are separated into two graphs for clarity. Error bars indicate the standard error.

METHODS. Muscimol paralysed all muscles in *C. elegans* including the body, enteric, egg-laying, and pharyngeal muscles (data not shown). There are no known GABAergic inputs to the pharyngeal muscles, and we are unsure whether the response of the pharynx to GABA is physiologically significant. We quantified sensitivity to muscimol by counting the pumping rate of the pharynx in increasing concentrations of muscimol. Between 4 and 12 worms of each genotype were placed on NG agar¹⁶ containing muscimol at the indicated concentration and seeded with bacteria. Pumping rates were scored after 30 min by directly counting pumps using a dissecting microscope.



Previous genetic analyses of neurotransmitter function have been based on screens for mutants resistant to specific neurotransmitter agonists or antagonists¹¹. By contrast, we identified mutants that phenocopy laser-operated animals lacking particular GABAergic neurons. In principle our approach could identify all genes that are not redundant in function and that are required specifically for GABA function in *C. elegans*. So far we have defined five such genes.

Some of the genes we have identified seem likely to encode familiar molecules. For example, *unc-25* probably encodes the GABA biosynthetic enzyme GAD, and *unc-49* might encode a GABA_A receptor subunit. Although these proteins are biochemically well characterized, the *in vivo* effects of mutations that affect such proteins have not previously been examined. For example, it has been proposed¹² that GABA has a neurotrophic role and is necessary for differentiation in early development. The rescue of AVL and DVB function with exogenous GABA in *unc-25* mutants indicates that GABA may not be necessary for the development of these neurons.

In addition, we have defined several other genes that seem likely to encode proteins that act in GABA presynaptic functions. The gene *unc-30* appears to be necessary for a number of aspects of D-type neuron synaptic development, including neurotransmitter expression and the specification of synaptic partners. The genes *unc-47* and *unc-46* are necessary presynaptically at all GABAergic synapses. These two genes appear to affect GABA release and might define components necessary for GABA vesicular loading, transport or fusion with the synaptic

membrane or differentiation of the neuromuscular junction. A molecular characterization of these genes might well define novel proteins involved in GABAergic neurotransmission. □

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The GABAergic nervous system of *Caenorhabditis elegans*

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γ -AMINO BUTYRIC acid (GABA) is the most abundant inhibitory neurotransmitter in vertebrates and invertebrates¹. GABA receptors are the target of anxiolytic, antiepileptic and antispasmodic drugs², as well as of commonly used insecticides³. How does a specific neurotransmitter such as GABA control animal behaviour? To answer this question, we identified all neurons that react with antisera raised against the neurotransmitter GABA in the nervous system of the nematode *Caenorhabditis elegans*. We determined the *in vivo* functions of 25 of the 26 GABAergic neurons by killing these cells with a laser microbeam in living animals and by characterizing a mutant defective in GABA expression. On the basis of the ultrastructurally defined connectivity of the *C. elegans* nervous system, we deduced how these GABAergic neurons act to control the body and enteric muscles necessary for different behaviours. Our findings provide evidence that GABA functions as an excitatory as well as an inhibitory neurotransmitter.

Of the 302 neurons in an adult *C. elegans* hermaphrodite⁴, 26 are stained by antibodies raised against the neurotransmitter GABA (Fig. 1). These 26 GABAergic cells comprise the six DD, 13 VD, four RME, one AVL, one DVB and one RIS neurons. As described later, we use laser microsurgery to identify roles in behaviour for all of the GABAergic neurons except RIS. We did

not observe any behavioural changes in animals in which RIS had been killed.

The DD and VD neurons are motor neurons that innervate the body muscles required for locomotion⁴ (Fig. 2a). *C. elegans* locomotion involves the propagation of a sinusoidal body wave from one end of the animal to the other. For example, if an animal is touched on the head it backs by producing a body wave of alternating contractions and relaxations of the opposing ventral and dorsal body muscles (Fig. 2b). By contrast, if an animal in which the DD and VD neurons have been killed by laser microsurgery is touched on the head, the animal simultaneously contracts its ventral and dorsal body muscles, resulting in a shrinkage in body length rather than in backward movement (Table 1a; Fig. 2c). Thus, the DD and VD neurons coordinate the wave of body muscle contractions involved in locomotion by preventing the simultaneous contraction of opposing muscles.

Is the role of the GABAergic DD and VD neurons in locomotion mediated by GABA? Mutations in the gene *unc-25* eliminate GABA expression in all 26 GABAergic neurons of *C. elegans*, probably by eliminating the GABA biosynthetic enzyme glutamic acid decarboxylase (ref. 5; and C. Johnson, personal communication). When touched on the head, *unc-25* animals shrink just like laser-operated animals that lack the DD and VD function (Fig. 2c; Table 1a), indicating that GABA is required for DD and VD function.

Can the known synaptic connectivity of the DD and VD neurons account for their function in locomotion? Based on the connectivities of these neurons, White *et al.*⁴ suggested that the DDs and VDs act as contralateral inhibitory neurons⁴. Electrophysiological studies by Stretton and co-workers^{6–9} of the larger parasitic nematode *Ascaris lumbricoides* have provided functional evidence for such a role for the *Ascaris* GABAergic motor neurons that correspond to the *C. elegans* VD neurons. Our studies of the behaviours of operated and mutant animals provide functional evidence for such a role for the *C. elegans* DD and VD neurons and strongly support the model that GABA is essential for contralateral inhibition. Specifically, it seems that the DD and VD motor neurons provide positive feedback to

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bending of the body by releasing GABA onto muscles that antagonize those that cause contraction. For example, when the VA and VB motor neurons cause the ventral muscles to contract, these neurons also excite the DD motor neurons, which in response release GABA and inhibit contraction of the dorsal muscles (Fig. 2a). The elimination of DD and VD neuron functions, either by selective killing with a laser or by mutation in *unc-25* animals, prevents this reciprocal inhibition and results in the simultaneous contraction of ventral and dorsal muscles. Our results indicate that the reciprocal inhibition provided by this circuit ensures that contraction of muscles on one side of the animal is linked to relaxation of muscles on the other side during locomotion.

The GABAergic RME neurons innervate muscles in the head necessary for foraging (Fig. 3a). During normal foraging the tip of the nose moves rapidly from side to side within a narrow arc of movement, as shown in Fig. 3b (J.K. and H.R.H., unpublished observations). Killing the four RME motor neurons by laser microsurgery resulted in worms with an abnormal foraging behaviour described as 'loopy' (Table 1b; Fig. 3c). Specifically, flexures of the nose became grossly exaggerated. Thus, the RMEs appear to limit the extent of head deflection during foraging. *unc-25* animals display the same defect as that produced by killing the RME neurons, suggesting that GABA is the relevant neurotransmitter (Table 1b; Fig. 3c).

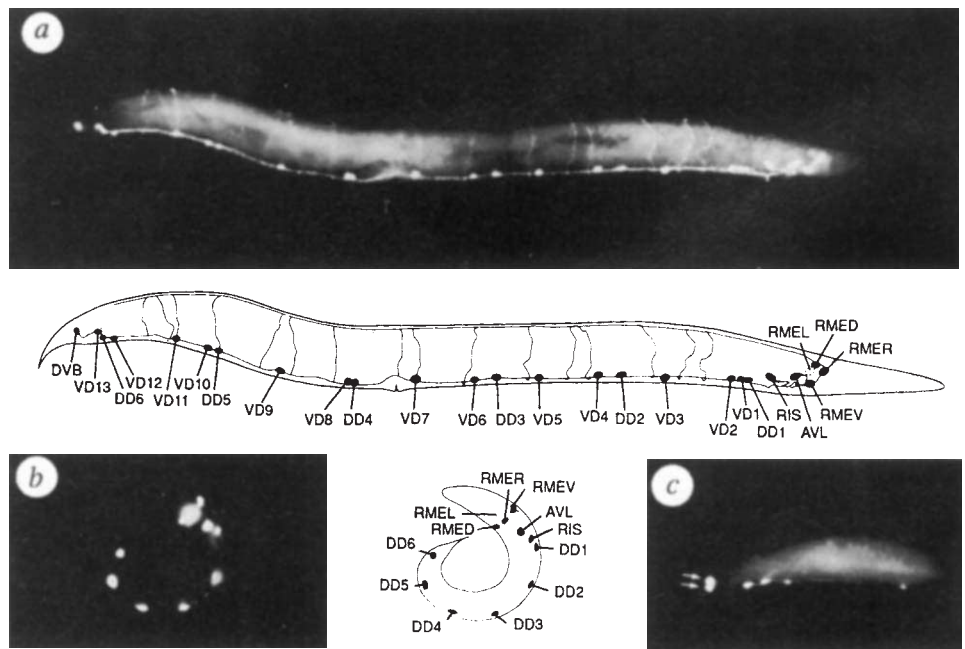
Does the connectivity of the RMEs explain the ability of these neurons to attenuate head deflections? The RMEs receive input from putative sensory neurons of the head and nose⁴ and, like the DDs and VDs, have output to contralateral muscles. For example, as shown in Fig. 3a, the RMED neuron receives input

from the SMBD neuron directly and from the SAAD neuron indirectly. Both of these neurons have long undifferentiated processes that run anteriorly and posteriorly along the dorsal side of the head, and White *et al.* proposed that these neurons provide proprioceptive information about head position⁴. Our results suggest that these putative sensory neurons might act through the RMEs: bending the head towards the ventral side might activate SAAD and SMBD as dorsal stretch receptors, which then activate RMED, which in turn relaxes the muscles on the ventral side of the head and restores a straightened head posture. Other sensory neurons might act through the RMEs in an analogous fashion.

The GABAergic motor neurons AVL and DVB each send a process to the enteric muscles (which consist of the intestinal, sphincter and anal depressor muscles)⁴, all of which contract during the defaecation cycle (Fig. 4a) (ref. 10). The defaecation cycle is initiated by a contraction of the posterior body muscles and is followed by a contraction of the anterior body muscles and then a contraction of the enteric muscles¹⁰. Killing AVL alone severely decreased the frequency of contractions of the anterior body muscles (Table 1c). Killing either AVL or DVB alone moderately decreased the frequency of enteric muscle contractions (Table 1d). Killing AVL and DVB together severely decreased the frequency of contractions of the enteric muscles, resulting in distention of the lumen of the gut (Fig. 4b, c). These results indicate that AVL and DVB are partially redundant in function and that these neurons are necessary for muscle contraction, indicating that they are stimulatory rather than inhibitory. *unc-25* mutants lack enteric muscle contractions, suggesting that GABA mediates this stimulatory function of AVL

FIG. 1 GABA immunoreactivity in *C. elegans*. a, Fluorescent photomicrograph and a tracing of the right side of a wild-type adult hermaphrodite stained with an antiserum raised against GABA. The names of the 26 cells stained with the anti-GABA antisera are indicated. Anterior is to the right, and dorsal is up. The pharynx is indicated by the dotted line. b, Fluorescent photomicrograph and tracing of a young L1 larva stained with an anti-GABA antiserum. Only the six DD neurons are stained in the ventral nerve cord and DVB is absent from the tail since this neuron is generated after the L1 stage¹⁷. c, Fluorescent photomicrograph of a *lin-4(e912)* animal stained with an anti-GABA antiserum. In *lin-4* mutants the mother of DVB undergoes an extra division and generates two DVB-like cells (arrowed)²⁴.

METHODS. Animals were stained with anti-GABA antisera²⁰ using a whole-mount procedure²¹. The GABAergic neurons were identified by the following criteria: (1) All cells in *C. elegans* have relatively invariant positions relative to other cells^{4,17}. The GABA immunoreactive cells occupy positions characteristic of particular classes of cells. The positions of immunoreactive cells were determined by comparison with the positions of other cells, as visualized with the nuclear stain 4-6-diamidino-2-phenylindole dihydrochloride (DAPI)²² (data not shown). (2) The process morphologies of the DD, VD and RME neurons are unique⁴ and matched those of the stained cells. (3) The number of the homologous members of each neuron class^{4,17} matched that of the stained cells. (4) The known time of generation of the neurons as identified¹⁷ was consistent with the stage at which these neurons could first be stained with anti-GABA antisera. (5) The AVL cell



body was identified based upon its known position relative to other cell nuclei (using Nomarski optics) and then killed with a laser microbeam; the operated animal was shown to lack the corresponding GABA-immunoreactive cell (data not shown). (6) *egl-5* and *lin-4* mutants, which are known to generate abnormal numbers of presumptive DVB neurons as a result of cell lineage defects^{23,24}, were found to have abnormal numbers of stained DVB-like neurons (data not shown). In the larger parasitic nematode *Ascaris lumbricoides*, there are cells with GABA immunoreactivity at positions and with morphologies similar to the DD, VD, RME and DVB neurons²⁵. It is unclear whether there are GABAergic homologues of AVL and RIS.

and DVB (Table 1d; Fig. 4c). Interestingly, *unc-25* mutants are not defective in anterior body contractions (Table 1c). One possibility for this difference between *unc-25* mutants and AVL-defective animals is that this function of AVL is mediated by a second neurotransmitter in this cell.

Do AVL and DVB directly excite the enteric muscles, or do

they stimulate the muscle contractions indirectly, for example by inhibiting an inhibitory neuron? All evidence suggests that DVB and AVL are primary exciters. First, the connectivity is consistent with this hypothesis: DVB synapses directly to the enteric muscles, AVL forms varicosities (which often indicate sites of transmitter release) at the enteric muscles, and no other neurons are known to innervate the enteric muscles^{4,11}. Second, killing

TABLE 1 The roles of GABAergic neurons

<i>a</i>				No. shrinking/ no. animals
Genotype	Cells killed		No. animals	
Wild type	None		21	0.00
Wild type	DD2-DD5, VD3-VD11		6	1.00
<i>unc-25</i> (GABA ⁻)	None		23	1.00
<i>b</i>				No. loopy foraging/ no. animals
Genotype	Cells killed		No. animals	
Wild type	None		12	0.00
Wild type	RMEV, RMEL, RMER, RMED		15	0.93
<i>unc-25</i> (GABA ⁻)	None		10	1.00
<i>c</i>				Anterior body contractions/ def. cycle
Genotype	Cells killed	No. animals	No. def. cycles	
Wild type	None	6	151	0.99
Wild type	AVL DVB	10	183	0.05
Wild type	AVL	14	153	0.03
Wild type	DVB	13	50	0.98
<i>unc-25</i> (GABA ⁻)	None	7	75	0.96
<i>d</i>				Enteric muscle contractions/ def. cycle
Genotype	Cells killed	No. animals	No. def. cycles	
Wild type	None	6	206	0.95
Wild type	AVL DVB	10	199	0.01 + 0.04*
Wild type	AVL	14	154	0.53
Wild type	DVB	13	143	0.71
<i>unc-25</i> (GABA ⁻)	None	7	76	0.12

Cells were killed using a laser focused through a Zeiss Axioplan microscope as previously described^{15,16}. All behaviours were assayed by studying 4-day-old wild-type (two days after surgery) or *unc-25(e156)* animals. a, The DD and VD neurons were killed in second stage (L2) larvae and were identified on the basis of their positions in the ventral nerve cord relative to other neurons¹⁷. Because we could not reliably identify the DD and VD neurons in the retrovesicular and the preanal ganglia, the cells DD1, DD6, VD1, VD2 and VD13 were not killed. Our analysis of animals double-stained with anti-GABA antisera and DAPI (data not shown), and reconstructions from electron micrographs¹⁸ indicate that although the ventral nerve cord is largely invariant in its order of neuron types, the positions of the VDs occasionally vary; therefore VDs might have remained in the mid-section of the ventral nerve cord in some operated animals. Based upon the degree of the observed variability, we estimate that fewer than one-third of our operated animals should have had more than one of the nine VDs in the ventral cord (VD3-VD11) remaining after surgery. b, Animals displaying exaggerated flexures of the head when moving forward were scored as 'loopy foraging'. The RMEs were identified in first-stage (L1) animals on the basis of their positions relative to other neurons¹⁹. Operated animals were scored for loopy foraging in a double-blind assay. c and d, The defaecation cycle (def. cycle) begins with a posterior body contraction (PBOC), which is followed about 4 s later by an anterior body contraction and an enteric muscle contraction¹⁰. Contractions were assayed by direct observation; in all operated animals the frequency of the defaecation cycle was normal (~45 s)¹⁰. The difference in the number of enteric muscle contractions between *unc-25* mutants and the AVL⁻ DVB⁻ animals might be a consequence of undetectable levels of GABA remaining in *unc-25* mutants or of a second neurotransmitter in these cells. Asterisk indicates that this value represents enteric muscle contractions that occurred between defaecation cycles, that is, more than 10 s following one PBOC and preceding the next. AVL and DVB were identified by position¹⁷ relative to other neurons in early L2 animals. Wild-type control animals, in which no cells were killed, were anaesthetized with the experimental animals but were not subjected to laser microsurgery. The following neurons were killed in various combinations without significantly reducing enteric muscle contractions: all neurons in the preanal ganglion, the lumbar ganglion, the retrovesicular ganglion, the ventral ganglion (other than AVL), the dorsal ganglion, the dorsal rectal ganglion (other than DVB) and those neurons in the lateral ganglia that send processes to the preanal ganglion, that is, AVA, AVB, AVD, AVH and AVJ.

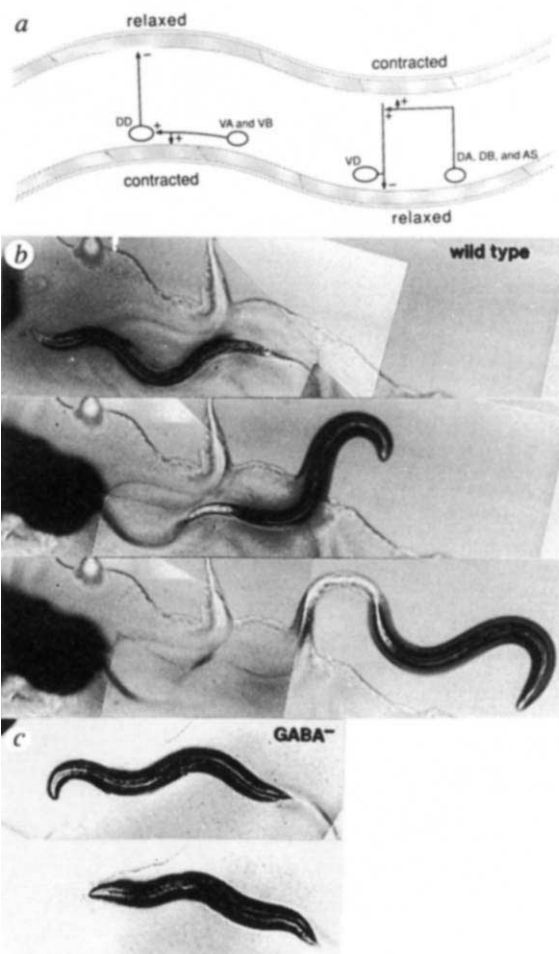


FIG. 2 The role of the DD and VD neurons in locomotion. a, Model for DD and VD neuronal function. Excitatory cholinergic motor neurons⁶ cause body muscle contractions: the VA and VB neurons cause contraction of ventral muscles, and the DA, DB and AS neurons cause contraction of dorsal muscles. These excitatory motor neurons also activate the GABAergic DD or VD neurons, which cause relaxation of the dorsal and ventral muscles, respectively. Presumptive excitatory synapses are designated by a plus sign, and inhibitory synapses are designated by a minus sign. Muscles are shaded. b and c, Locomotory behaviour in b, a wild-type animal, and c, an *unc-25(e156)* mutant, which lacks GABA expression⁵. The head of each animal was touched with a platinum wire (shadow to the left of the wild type) and photographed at intervals of ~1 s. Touch to the head of the wild-type animal resulted in backward movement characterized by deep sinusoidal waves. Touch to the head of the *unc-25* mutant caused the ventral and dorsal muscles to contract simultaneously, resulting in no substantial backward movement, reduced flexures of the body and a shrinkage of body length. *unc-25* animals are still capable of some forward movement (not shown), although the amplitude of the body flexures is greatly reduced.

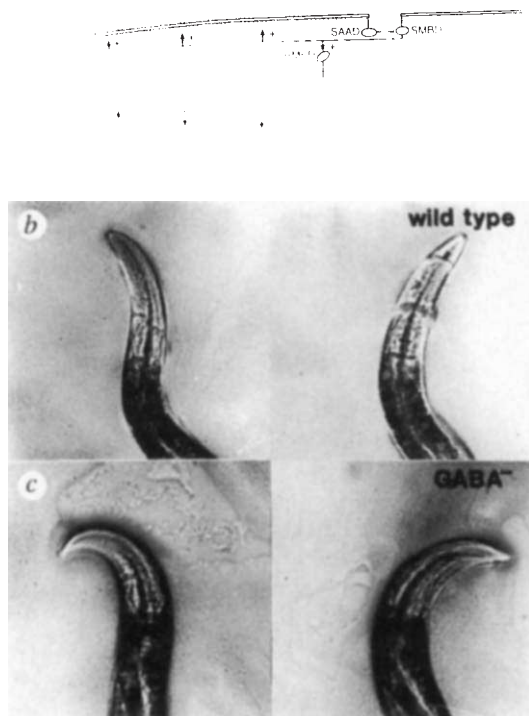
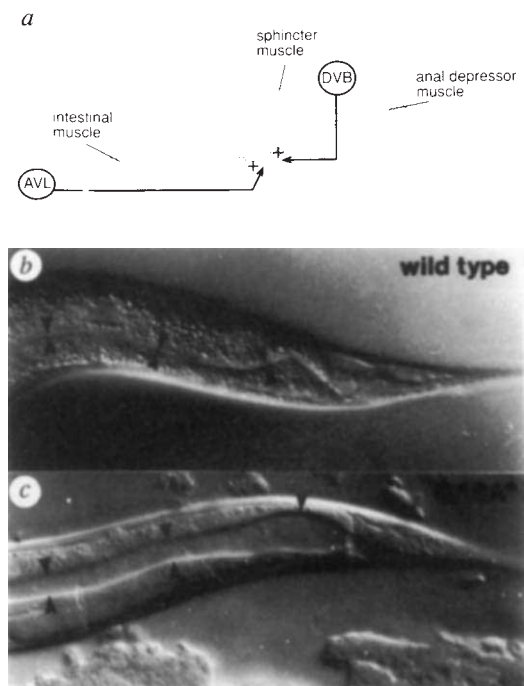


FIG. 4 The role of AVL and DVB in enteric muscle contractions. **a**, Model for AVL and DVB neuronal function. The four enteric muscles involved in expulsion comprise the anal depressor, the sphincter, and the two bilaterally symmetric intestinal muscles⁴. These muscles are connected by gap junctions and send arms to the preanal ganglion, where DVB synapses to the intestinal muscles. The sphincter appears to be relaxed before expulsion, allowing filling of the rectum; it then contracts with the anal depressor and intestinal muscles to prevent reflux of the rectal contents into the gut (our unpublished observations). Although AVL has not been observed to form synapses with enteric muscles, its process runs closely apposed to the enteric muscles and forms gap junctions with DVB. The AVL cell body is located far anteriorly in the ventral ganglion of the head. **b** and **c**, The anal region **b**, of a wild-type animal, and **c**, of an *unc-25(e156)* GABA-deficient mutant. Note that the lumen of the gut (arrowheads) is greatly distended in the *unc-25* worm as a consequence of abnormal enteric muscle function.

FIG. 3 The role of the RME neurons in foraging. **a**, Model for RME neuronal function. There are four RME neurons: RMEV, RME, RMEL and RMER. Based upon the anatomically-defined connectivity of the *C. elegans* nervous system⁴, each RME neuron receives inputs from a variety of sensory neurons. For example, the dorsal RME (RMEV) receives inputs from the putative stretch receptor neurons SAAD and SMBD as well as from the putative mechanosensory neurons OLQ, OLL, IL1 and IL2. For simplicity, this figure illustrates only the inputs from SAAD and SMBD; inputs from the other sensory neurons could function analogously. The circuit of RMEV is similar to that of RME. In addition, RMEV and RME have long undifferentiated processes in the dorsal and ventral nerve cords; these processes might act as sensory endings on their own (not shown). RMEL and RMER are components of analogous circuits on the left and right sides, but these neurons do not receive input from the SMBs. $\vdash$, Gap junction; arrows, chemical synapses; unfilled bars, sensory processes. **b** and **c**, Foraging in **b**, a wild-type and **c**, an *unc-25(e156)* mutant. Lateral deflections of the nose and head are very restricted in wild-type animals but are very sweeping in *unc-25* animals, which lack GABA.



all other neurons with processes in the vicinity of the enteric muscles did not cause defects in defaecation (Table 1 legend). Third, we have genetic and pharmacological evidence that the inhibitory and stimulatory activities of GABA are mediated by distinct receptors (ref. 5; and E.J., unpublished results). Although GABA is generally thought to have an inhibitory effect on postsynaptic membranes¹², our results suggest that GABA has both inhibitory and excitatory effects in *C. elegans*.

How might GABA direct excitation? GABA_A receptor activation in vertebrates directs inhibition by maintaining a negative membrane potential as a result of an inward Cl⁻ conductance. It is possible that excitatory effects of GABA are mediated by an outward Cl⁻ conductance through a GABA_A receptor, leading to

membrane depolarization; such an effect of GABA has been proposed to occur in the developing mammalian brain¹³. Alternatively, GABA might be acting directly on a novel cationic channel or indirectly on such a channel through a G-protein-linked receptor such as the GABA_B receptor¹⁴, and so cause depolarization as a consequence of cation influx.

To understand the neuronal and neurochemical bases of behaviour, it is necessary to integrate behavioural studies with anatomical, biochemical and physiological analyses of the nervous system. Here we have identified all GABA-immunoreactive neurons present within an entire nervous system. Each of these neurons is known in terms of its developmental ancestry and its synaptic connectivities. Although GABA is usually considered

to be an inhibitory neurotransmitter, our data provide evidence that GABA can have excitatory as well as inhibitory effects on postsynaptic membranes. Our observations reveal how a specific neurotransmitter can act in known neural circuits to control the behaviour of an intact, living animal. □

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Mechanosensitive channels transduce osmosensitivity in supraoptic neurons

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VASOPRESSIN is a peptide hormone synthesized by neurons of the supraoptic and paraventricular nuclei, which project axon terminals to the neurohypophysis. Consistent with its antidiuretic properties, vasopressin release rises as a function of plasma osmolality^{1–3}, a response that results from accelerated action potential discharge^{4–6}. Previous studies have shown that increases in fluid osmolality depolarize supraoptic neurons in the absence of synaptic transmission^{7,8}, suggesting that these cells behave as intrinsic osmoreceptors. The mechanism by which changes in osmolality are transduced into an electrical signal is unknown, however. Here we report that changes in cell volume accompany physiological variations in fluid osmolality and that these modulate the activity of mechanosensitive cation channels in a way that is consistent with the macroscopic regulation of membrane voltage and action potential discharge. These findings define a function for stretch-inactivated channels in mammalian central neurons.

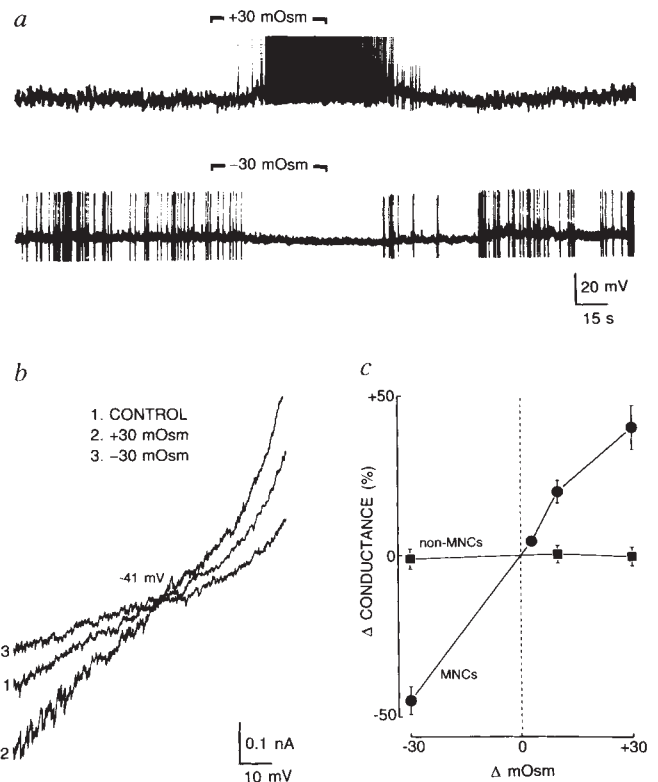


FIG. 1 *a*, Whole-cell voltage recordings from isolated supraoptic MNCs. Superfusion of a hypertonic solution (+30 mOsm) provoked a reversible depolarization and increased spike discharge (full amplitude not shown). In contrast, application of a hypotonic solution (−30 mOsm) induced membrane hyperpolarization and inhibited spike discharge. The traces in *b* show current responses to voltage ramps (16 mV s^{−1}) recorded from a supraoptic MNC. Application of hypertonic (2) and hypotonic solutions (3), respectively, increased and decreased slope conductance compared to control (1) and wash (not shown). Note that all three current ramps intersect near −41 mV. The graphs in *c* plot mean (± s.e.m.) changes in conductance (as per cent of control) evoked in MNCs (*n*=19; filled circles) and non-MNCs (*n*=13; filled squares) upon application of various osmotic stimuli.

METHODS. Petri dishes containing cells prepared as described⁹ were perfused with a solution (295 ± 2 mOsm; pH 7.4) comprising (mM): NaCl (140), KCl (3), CaCl₂ (1), MgCl₂ (1), HEPES (10) and mannitol (30). Osmolality was adjusted by adding or removing mannitol as required. All voltage-clamp measurements were made in the presence of tetrodotoxin (TTX; 1 μM). Recordings (at 22–25 °C) were obtained either from MNCs (diameter >15 μm)⁹ or from non-MNCs using the whole-cell configuration of the patch-clamp technique¹⁷. Non-MNCs included neighbouring non-neuroendocrine cells (diameter <10 μm)⁹ isolated from the same blocks of tissue used to obtain MNCs, as well as larger neurons similarly isolated from temporal cortex, cerebellum, or from the hypothalamic ventromedial and arcuate nuclei. Both oxytocin¹⁸ and vasopressin^{2,3} are released in response to hypertonic stimulation in the rat, which is in agreement with the apparent osmosensitivity of both types of MNCs⁴. Therefore, no attempt was made to characterize the cells beyond their identification as MNCs. Recording electrodes (2–5 MΩ) were filled with a solution (pH 7.15) comprising (mM): KCl (150), MgCl₂ (1), HEPES (10), EGTA (1.6), Na₂-ATP (1.5) and cAMP (0.2). Membrane currents (DC; 200 Hz) were digitized by a lab-master interface and analysed using pCLAMP software.

Patch-clamp recordings were obtained from magnocellular neurosecretory cells (MNCs) acutely isolated from the supraoptic nuclei of adult rats⁹. Bath-application (0.5–10 min) of hypertonic solutions evoked a sustained and reversible membrane depolarization accompanied by increased firing in each of 24 MNCs tested (Fig. 1*a*). Conversely, reducing the osmolality

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(mOsm) of the perfusate hyperpolarized and inhibited firing in 10 of 14 MNCs (Fig. 1a). Voltage-clamp analysis (Fig. 1b) indicated that voltage responses elicited by increases or decreases in osmolality resulted from the enhancement or suppression, respectively, of a current reversing at -41.5 ± 2.6 mV ($n = 36$). This value shifted to -90.3 ± 2.2 mV upon removal of external Na^+ ($n = 9$), was increased upon raising $[\text{K}^+]_o$ (see below), but was unaffected by replacement of 94% of the external Cl^- with SO_4^{2-} (-41.0 ± 1.6 mV; $n = 3$), indicating that the conductance discriminates poorly between monovalent cations. Consistent with a role in mediating a specific intrinsic osmosensitivity, the data shown in Fig. 1c reveal a sensitive relation between changes in input conductance (G_i), due to a modulation of the cationic conductance, and external osmolality in MNCs ($+1.48\%G_i$ per mOsm; $R = 0.97$; $n = 19$), but not in non-MNCs ($+0.05\%G_i$ per mOsm; $R = 0.98$; $n = 13$).

As changes in cell volume are frequently associated with variations in external fluid osmolality¹⁰, the latter have been proposed to participate in the cellular transduction of osmotically mediated effects¹. In agreement with this, Fig. 2 shows that a reversible decrease in cell volume is associated with the conductance increase evoked by hypertonic stimulation of MNCs. Linear regression analysis of the data confirms a tight temporal correlation between volume and conductance changes associated with the onset and offset of the response ($R = 0.88$; $n = 29$). Figure 2 also shows that equivalent osmotically evoked shrinkage observed in non-MNCs is not associated with conductance changes.

To determine whether decreases in cell volume suffice to activate the transduction mechanism, negative pressure was applied to the inside of whole-cell patch pipettes to produce changes in

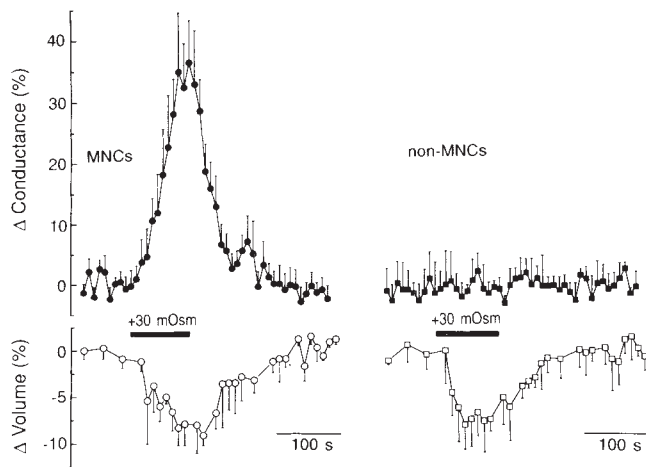


FIG. 2 Top traces show average changes ($\pm$ s.e.m.) of input conductance (filled symbols) recorded from MNCs (circles; $n = 7$) and non-MNCs (squares; $n = 8$) upon exposure to a hypertonic stimulus. Bottom traces plot the corresponding changes in cell volume (per cent of control; open symbols) estimated from 6 MNCs and 5 non-MNCs exposed to an identical stimulus.

METHODS. At each time point indicated, the per cent change of input conductance ($\% \Delta G_i$) was calculated as $\% \Delta G_i = 100 \times [(I_t - I_o)/I_o]$, where I_o is the mean current response to -20 mV steps applied during the pre-stimulus period ($V_h = -70$ mV), and I_t is the membrane current response to the same step applied at time t . Per cent change in cell volume ($\% \Delta V_t$) was estimated as $\% \Delta V_t = [(CSA_o)^{3/2} - (CSA_t)^{3/2}] / [(CSA_o)^{3/2}]$, on the assumption that the cells were ellipsoids whose size changed uniformly in all planes¹⁹, where CSA_t is the maximal cross-sectional area of a cell measured from a digitized image obtained using a Leitz scanning laser microscope. Relative changes in cell volume are expressed as per cent of the mean cross-sectional area (CSA_o) observed during a 5-min control period.

cell volume comparable to those elicited by hypertonic stimulation. As shown in Fig. 3a, 7 of 9 current-clamped MNCs responded to this stimulus with a reversible depolarization and accelerated firing. Under voltage-clamp (Fig. 3b), the same procedure elicited a current reversing at -42.3 ± 2.0 mV ($n = 4$), a value which corresponds to that measured for the osmotically evoked current. Furthermore, upon increasing $[\text{K}^+]_o$ to 6 mM, the reversal potentials of the pressure- and osmotically evoked currents, respectively, shifted to -10.3 ± 3.4 mV and -11.0 ± 2.9 mV (Fig. 3b; $n = 3$). Finally, in each of two cells tested, application of negative pressure reversed a decrease of conductance which had been induced by the prior application of a hypotonic solution. These data suggest that responses of MNCs to changes in pipette pressure or external osmolality result from the modulation of the same population of non-selective cationic channels.

Cell-attached recordings obtained from 53 of 78 patches on MNCs revealed unitary currents reversing at -41.5 ± 4.5 mV (Fig. 4a) carried through channels displaying a mean conductance of 31.8 ± 1.9 pS (Fig. 4b). As illustrated in Fig. 4c, increasing external osmolality by $+30$ mOsm enhanced overall channel activity ($n = 11$ patches). A similar effect could also be induced by applying small amounts of pressure (± 0.3 – 1.8 cm H_2O) to the inside of the recording pipette (Fig. 4c), suggesting that the ionic channels involved are mechanosensitive^{11,12}. But if a direct mechanical gating of such channels is to account for our observations, then the activity of these channels should diminish as cellular volume increases. The data in Fig. 4d, e show that from a minimal value measured at negative pressure, small increments in pipette pressure initially enhanced, but subsequently inhibited, channel activity (Fig. 4d). The existence of a bell-shaped relationship between activity and pipette pressure (Fig. 4e) is an established feature of stretch-inactivated channels¹³ which indicates that the frequency of channel openings is lowest when the membrane is distended but increases as tension relaxes (during cell shrinkage, for example).

Although a variety of mechanosensitive ion channels have been found in neurons^{11,14}, their possible function in these cells

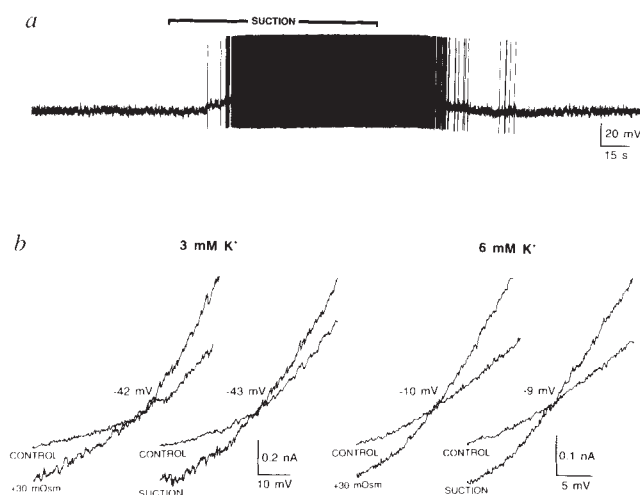


FIG. 3 a, Voltage response of an isolated supraoptic MNC ($V_h = -61$ mV) to the application of mild suction (bar; ~ 1.5 cm H_2O) to the interior of the patch pipette during a whole-cell recording. b, Membrane current responses to voltage ramps (16 mV s^{-1}) applied before (control) and during hyperosmotic ($+30$ mOsm) stimuli, or during suction-evoked volume decreases (about -10% , estimated visually). The left-hand panels show that in the presence of 3 mM $[\text{K}^+]_o$, these stimuli elicit currents which reverse at comparable membrane potentials. The right-hand panel shows that in the presence of 6 mM external $[\text{K}^+]_o$, the observed reversal potentials both shift in the positive direction.

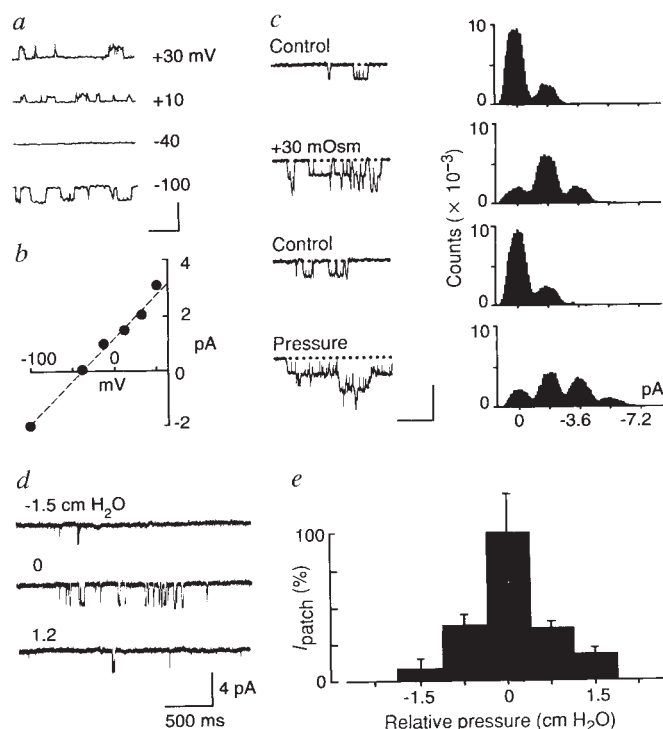


FIG. 4 *a*, Recordings from a cell-attached patch held at various potentials in the presence of hypertonic saline (+30 mOsm). Calibration bars, 4 pA and 50 ms. *b*, *I*-*V* relation for the channel shown in *a*. Dotted line is a linear regression fit through the data points ($R=0.99$), where slope conductance is 32.3 pS and the reversal potential is -39.2 mV. *c*, Excerpts of channel currents recorded from another patch ($V_h = -100$ mV) containing at least three channels, under the conditions indicated (dotted line, closed state; calibration, 4 pA and 200 ms). Corresponding all-points amplitude histograms (right) reflect the enhancement of channel activity induced by hypertonic stimulation of the cell or by application of pressure to the membrane patch. *d*, Recordings from an on-cell membrane patch ($V_h = -110$ mV) at the pipette pressures indicated. *e*, Effects of pressure on channel activity expressed as the mean ($\pm$ s.e.m.) of the average total patch current (I_{patch}) recorded from 7 cells. As the residual pipette pressure is frequently non-zero when open to the atmosphere¹¹, zero pressure was arbitrarily defined as that corresponding to maximal I_{patch} .

METHODS. Cell-attached recordings¹⁵ were obtained using pipettes containing (in mM): sodium glutamate (140), KCl (3), tetraethylammonium-chloride (TEA-Cl) (6), 4-aminopyridine (4-AP) (4), CdCl₂ (0.01), CsCl (4), EDTA (1), HEPES (10), TTX (0.002), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (0.2), D-amino-5-phosphonopentanoate (APV) (0.2), pH 7.35. Currents were filtered at 2 kHz (4-pole Bessel filter), displayed on chart paper, or digitized at 10 kHz. Membrane potentials were estimated as the mean membrane potential (V_m) minus the voltage command applied to the pipette. Mean values of V_m were obtained as zero current points during whole-cell voltage ramps applied in control (-62 mV) or hypertonic solutions (-57 mV). Average total patch current was calculated as the charge carried through all channels within a membrane patch, divided by the duration of the corresponding recording period. The values plotted in *e* are normalized relative to the maximum values obtained in each patch.

has remained elusive¹⁵. Our findings suggest that changes in membrane tension associated with osmotically evoked volume changes directly modulate the activity of stretch-inactivated cationic channels to confer upon hypothalamic MNCs an intrinsic osmosensitivity which is important for the control of vasopressin release¹⁶. □

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Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor

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MACROPHAGES interact with other cells and components of the extracellular environment by means of adhesion receptors^{1,2}. Adhesion to artificial substrata *in vitro* facilitates isolation of macrophages³, and has been used to generate antibodies that inhibit their migration *in vivo*^{4,5}. Unlike other cell types, macrophages attach to tissue culture plastic in the absence of divalent cations. Here we use an adhesion assay exploiting this property to isolate a rat monoclonal antibody, 2F8, which totally inhibits divalent cation-independent adhesion of murine macrophages to tissue culture plastic in the presence of fetal calf serum. Immunoprecipitation from macrophages and stably transfected Chinese hamster ovary cells revealed that the antigen recognized by monoclonal 2F8 is identical to murine macrophage scavenger receptor^{6,7}. We propose a novel function for this molecule, previously described as an endocytic receptor, thus providing a mechanism for mononuclear phagocyte recruitment to and retention in ligand-rich tissues such as in atherosclerotic lesions.

To define novel macrophage (MΦ) adhesion molecules, we investigated the adhesion of these cells to plastic substrata. Murine MΦ adhesion to bacteriological plastic in the presence of serum is dependent on divalent cations and is mediated by the β₂ integrin, complement receptor type 3 (CR3)⁴. The rat monoclonal antibody 5C6 inhibits this adhesion, but has little effect on MΦ adhesion to tissue-culture-treated plastic surfaces⁴. On this surface and in the presence of either EDTA or antibody 5C6, MΦ lose their spread morphology but remain adherent. By focusing on this EDTA-resistant adhesion, our strategy was designed to bias the investigation away from the well characterized divalent cation-dependent integrin⁸ and selectin^{9,10} families of adhesion molecules.

The RAW264 cell line expresses many features of mature murine MΦ^{11,12} and has similar *in vitro* adhesion properties to

those of primary thioglycolate-elicited peritoneal M Φ (TPM Φ). Spleen cells from an albino Oxford (AO) rat immunized four times with RAW264 cells were fused with the Y3 rat myeloma line as described¹³. Supernatants were tested for their ability to inhibit RAW264 cell adhesion to tissue culture plastic multiwell plates in the presence of both fetal calf serum and 5mM EDTA. In the presence of EDTA, M Φ adhesion was decreased to ~50–60% of control (Fig. 1). A single hybridoma (from >600 tested) produced monoclonal antibody 2F8, a rat IgG2b that totally inhibits EDTA-resistant adhesion without compromising cell viability, or inducing cell aggregation or detectable morphological changes. Isotype-matched control antibodies (including 5C6) did not inhibit this adhesion.

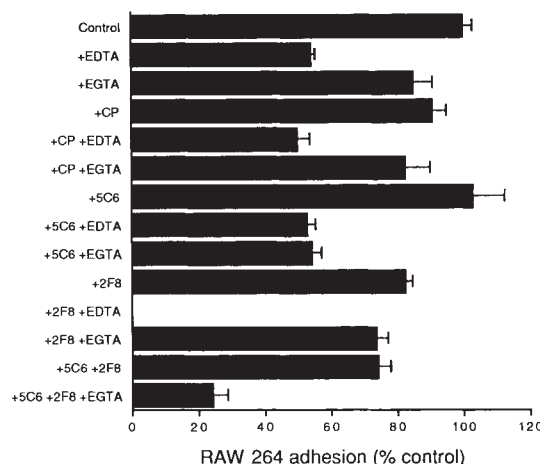
The monoclonal antibody 2F8 was unable to inhibit M Φ adhesion in the presence of divalent cations, even when antibody 5C6 was also present to inhibit CR3. When EGTA was used to chelate Ca²⁺, antibodies 2F8 and 5C6 partially but reproducibly inhibited adhesion. The residual Ca²⁺-independent adhesion suggests that there is a third, non-CR3-,

non-2F8-mediated adhesion system for murine M Φ which we did not investigate further. It is not yet clear whether 2F8-mediated adhesion has an absolute requirement for the absence of divalent cations, or if it only becomes significant when other adhesion molecules have been functionally disabled by the chelation of divalent cations. Under serum-free conditions, antibody 2F8 did not inhibit EDTA-resistant adhesion to tissue-culture-treated plastic (just as antibody 5C6 did not inhibit adhesion to bacteriological plastic in the absence of fetal calf serum⁴). Assays were routinely done in the presence of fetal calf serum, and 2F8-inhibitable adhesion was also demonstrated by precoating tissue-culture-treated plastic surfaces with fetal calf serum before serum-free adhesion assays (results not shown). This suggests that the adhesion-promoting ligand of the 2F8 antigen is a constituent of fetal calf serum and is immobilized on tissue-culture-treated plastic, but not bacteriological plastic surfaces.

While the RAW264 cell line was used as the immunogen, 2F8-inhibitable adhesion was also demonstrated with primary

FIG. 1 Adhesion of RAW264 cells to tissue-culture-treated plastic (TCP) surfaces.

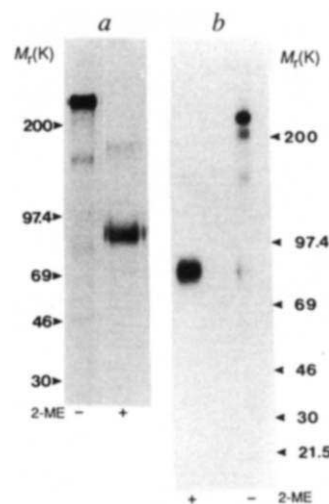
METHODS. RAW264 cells were grown on bacteriological plastic (BP) (Greiner Labortechnik Ltd) in RPMI plus 10% FCS, harvested with 5mM EDTA in PBS and washed in FCS-containing RPMI. Adhesion was assayed as described⁴. Cells were washed and suspended in HEPES-buffered RPMI with 10% FCS and plated at a density of 10⁵ cells per well in 96-well flat-bottomed TCP plates (Falcon, Becton Dickinson). EDTA or EGTA was added to a final concentration of 5mM where indicated. Purified monoclonal antibodies (mAbs) were used at a final concentration of 2 μ g ml⁻¹: mAbs 2F8 and 5C6⁴ were purified from rat ascites in our laboratory; CAMPATH-1G (CP)¹⁸ (an isotype-matched mAb which is unreactive against mouse cells) was a gift from G. Hale and H. Waldmann. After incubation for 30 min (+/-mAb, +/-chelator) at 4 °C, cells were placed at 37 °C for a further 90 min to allow adhesion, then washed three times in PBS. In some assays, cell viability was assessed at each step by trypan-blue exclusion. After observing adherent cells under phase-contrast microscopy, cells were fixed in methanol to enable them to be stained and quantified⁴. Briefly, after staining with 40% (v/v) Giemsa solution for 1 h, plates were washed in tap water, dried, and the retained dye solubilized in methanol. Stain was quantified by measuring absorbance at 450nm in an automatic plate reader (Anthos HTII, Denley Instruments). Results are expressed as a percentage of control adhesion (mean $\pm$ s.d.) were



derived from a single experiment ($n=6$) and are representative of >10 similar such experiments. Parallel experiments with TPM Φ revealed similar adhesion characteristics (not shown).

FIG. 2 Immunoprecipitation of 2F8 antigen from RAW264 cells labelled metabolically with ³⁵S-TRANS (a), or at the cell surface with ¹²⁵I (b).

METHODS. RAW264 cells were grown to confluence in 35-mm TCP dishes (Nunc, Denmark) in RPMI plus 10% before metabolic labelling (a). They were washed in methionine-free DMEM, preincubated in the same medium containing 10% dialysed FCS for one hour, after which 50 μ Ci ml⁻¹ of ³⁵S-TRANS label (ICN, UK) was added and cells incubated for a further 16 h until lysis and immunoprecipitation. In (b), 2 $\times$ 10⁷ RAW264 cells were labelled with ¹²⁵I (Amersham) by an exogenous H₂O₂-lactoperoxidase technique¹⁹. After labelling (in a and b), cells were washed three times in PBS and then lysed on ice in 150 mM NaCl, 10mM EDTA, 10mM Na₂S₂O₈, 10 mM Tris (pH 8.0), 1mM PMSF, 5mM iodoacetamide and 1% NP-40. Lysates were spun for 5 min in a microcentrifuge, and then at 100,000g to remove debris. Lysates were precleared with 25 μ g ml⁻¹ CAMPATH-1G followed by protein G-Sepharose (Pharmacia LKB) precipitation at 4 °C. After incubation with 25 μ g ml⁻¹ purified 2F8, the antigen was precipitated with protein G-Sepharose, washed six times in 10mM Tris, pH8, buffers at 4 °C (3 times in 500 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.05% (w/v) SDS; twice in 150 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.05% (w/v) SDS; once in 0.05% (w/v) SDS, then boiled in Laemmli gel buffer in the presence (+) or absence (-) of 5% (v/v) 2-mercaptoethanol (2-ME) before separation by 5–10% SDS-PAGE²⁰. Gels were fixed and (in a) impregnated in EN³HANCE (Du Pont) before drying and exposure to film at -70 °C.



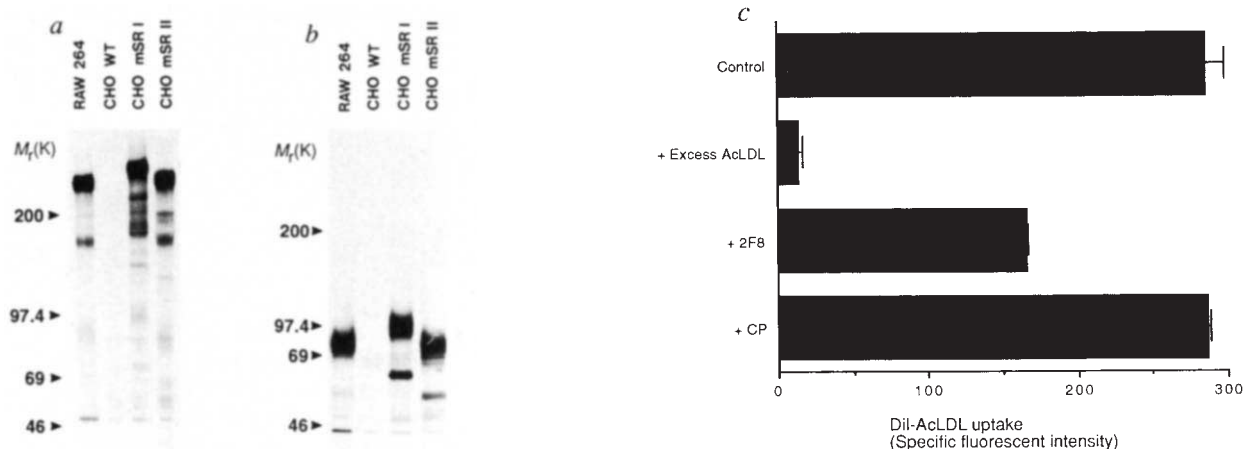


FIG. 3 Recognition of murine scavenger receptor by mAb 2F8. Immunoprecipitation with mAb 2F8 from metabolically labelled cells (a, b). Inhibition by mAb 2F8 of uptake of Dil-labelled AcLDL by RAW264 cells (c).

METHODS. CHO cells transfected with either murine scavenger receptor type I (mSRI) or type II (mSR II) and untransfected wild-type CHO cells (WT)¹⁵ were all gifts from M. Krieger. Cells were grown to confluence in 35-mm TCP dishes, metabolically labelled, and immunoprecipitated with mAb 2F8 as for Fig. 2a. Precipitates were separated by 5–10% SDS-PAGE in the absence (a) or presence (b) of β -mercaptoethanol before exposure to film at -70°C . Uptake of Dil-AcLDL (c) was measured as described²¹. RAW264 cells were plated at 10^6 cells per well in 24-well TCP plates. After overnight incubation in RPMI plus 5% FCS, cells were washed, and incubated in the same medium with $4\text{ }\mu\text{g ml}^{-1}$ Dil-AcLDL (Biogenesis Ltd)²² plus either $400\text{ }\mu\text{g}$

ml^{-1} AcLDL (excess AcLDL), or $10\text{ }\mu\text{g ml}^{-1}$ mAb 2F8 (+2F8) or CAMPATH-1G (+CP). After 3 h incubation at 37°C , cells were washed four times in PBS containing 0.1% (w/v) bovine serum albumin and 10 mM $\text{Na}_2\text{S}_2\text{O}_8$, and 3 times in PBS alone. Cells were recovered by pipetting after incubation in PBS containing 5mM EDTA and 15mM lidocaine-HCl (Astra)²³, fixed in 3% (w/v) paraformaldehyde in PBS, and subjected to flow cytometry (FACScan, Becton Dickinson) using excitation at 488 nm and collection of emitted fluorescence at 563–607nm (FL2 photomultiplier). Specific fluorescence intensity was calculated by subtracting autofluorescence intensity from fluorescence intensity of Dil-AcLDL-labelled cells using geometric means derived from data analysis by Lysis II software (Becton-Dickinson). Results (mean $\pm$ s.d.) are from a single experiment done in triplicate and are representative of two independent experiments.

TPM Φ (not shown). Immunochemical analysis by flow cytometry and histology revealed a 2F8 expression pattern restricted to subpopulations of mononuclear phagocytes (D.H., I.F. and S.G., manuscript in preparation). No non-M Φ cells or cell lines tested expressed 2F8 antigen or displayed EDTA-resistant adhesion as defined by this assay (data not shown).

Immunoprecipitation following either cell-surface or metabolic labelling showed the 2F8 antigen migrating as a single, diffuse band under reducing SDS-PAGE with $M_r \approx 90$ (Fig. 2). Under non-reducing conditions, the molecule appeared as disulphide-linked multimers, with the largest band predominating. The apparent M_r of these ($\sim 156\text{K}$ and $\sim 238\text{K}$, with an extra band at $\sim 206\text{K}$ that was consistently present in cell-surface-labelled preparations) did not represent numerically exact multimers, but may have been due to aberrant migration in the multimerized state because of extensive glycosylation of the monomer (deglycosylated monomer has an M_r of 41K (not shown)), and/or because of intrachain disulphide bonds. As this appearance was suggestive of the M Φ scavenger receptor¹⁴, Chinese hamster ovary (CHO) cells stably transfected with murine M Φ scavenger receptor complementary DNAs¹⁵ were metabolically labelled before immunoprecipitation with antibody 2F8 (Fig. 3a and b). Untransfected wild-type CHO cells were unable to take up acetylated low-density lipoprotein (AcLDL) labelled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (not shown) and revealed no 2F8-immunospecific bands. Scavenger receptor transfectants took up DiI-labelled AcLDL to the same extent as did RAW264 cells, and following immunoprecipitation, revealed 2F8-specific bands of the appropriate M_r for scavenger receptor. Although the type-I receptor contains a 102-amino-acid domain which is absent from the type-II receptor⁷, both forms are recognized by antibody 2F8. The receptor precipitated from RAW264 cells migrated with the same mobility as the type-II receptor. To

characterize the functional activity of 2F8 further, its ability to inhibit the uptake of AcLDL by RAW264 cells in the presence of divalent cations was tested (Fig. 3c). At a concentration of $10\text{ }\mu\text{g ml}^{-1}$, antibody 2F8 partially, but specifically and reproducibly, inhibited the uptake of DiI-AcLDL.

The data show that the M Φ scavenger receptor, like CR3, can mediate both adhesion and endocytosis. As in the case of CR3, the component in fetal calf serum that mediates 2F8-dependent adhesion of M Φ *in vitro* is not known. It is of interest that β_2 -integrins seem to mediate leukocyte adhesion by binding denatured proteins¹⁶. Scavenger receptors bind with high affinity to a range of ligands, including chemically modified lipoproteins⁶, but which, if any, of these are found in serum requires further study. We propose that mononuclear phagocytes may use the scavenger receptor as an adhesion molecule under both physiological and pathological conditions, especially where known scavenger receptor ligands are abundant, such as in atherosclerotic plaques¹⁷. The strategy outlined here, together with the specific tools to inhibit known adhesion molecules, will facilitate further functional studies on the scavenger receptor, and enable additional M Φ adhesion molecule(s) to be identified. $\square$

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Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein

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TETANUS toxin inhibits neurotransmitter release by selectively blocking fusion of synaptic vesicles^{1,2}. Recently tetanus toxin was shown to proteolytically degrade synaptobrevin II (also named VAMP-2), a synaptic vesicle-specific protein^{3,4}, *in vitro* and in nerve terminals^{5,6}. As targets of tetanus toxin, synaptobrevins probably function in the exocytotic fusion of synaptic vesicles. Here we describe a new synaptobrevin homologue, cellubrevin, that is present in all cells and tissues tested and demonstrate that it is a membrane trafficking protein of a constitutively recycling pathway. Like synaptobrevin II, cellubrevin is proteolysed by tetanus toxin light chain *in vitro* and after transfection. Our results suggest that constitutive and regulated vesicular pathways use homologous proteins for membrane trafficking, probably for membrane fusion at the plasma membrane, indicating a greater mechanistic and evolutionary similarity between these pathways than previously thought.

There are two types of vesicular trafficking pathways in eukaryotic cells that recycle via the plasma membrane: constitutive pathways, with continuous exo- and endocytosis of membranes, and regulated pathways, with triggered exocytosis followed by rapid endocytosis. These pathways are thought to be fundamentally different^{7,8} but there are similarities^{9,10}. Both pathways use clathrin-coated pits for endocytosis. Synaptic vesicle biogenesis seems similar to that of receptor-carrying vesicular organelles¹¹, and polarized epithelia have a polarization in their vesicular trafficking pathways analogous to neurons¹². Finally, expression in fibroblasts of synaptophysin, another synaptic vesicle-specific protein, results in the targeting of this protein into the receptor-

mediated endocytosis pathway in the transfected cells^{13–15}. These findings suggest that the pathways of receptor-mediated endocytosis and synaptic vesicles are related but do not demonstrate the direct analogy or homology that we have now established.

We screened a genomic library for genes encoding synaptobrevins and isolated a partial genomic clone encoding a novel sequence distinct from, but homologous to, synaptobrevins. Using specific probes from this sequence, full-length complementary DNA clones were isolated from rat brain (Fig. 1a). Alignment of the translated amino-acid sequence of these clones with the sequences of synaptobrevins demonstrates that the new sequence is a novel form of synaptobrevin with a high degree of sequence homology with other synaptobrevins (59% sequence identity between all mammalian forms; Fig. 1b). The synaptobrevins, small and highly conserved proteins, are among the most abundant synaptic vesicle proteins^{3,4} and are the targets of clostridial neurotoxins in nerve terminals^{5,6}. Because clostridial toxins inhibit synaptic vesicle fusion but not docking, their action via synaptobrevins suggests that synaptobrevins function in the fusion of synaptic vesicles with the presynaptic plasma membrane^{1,2,16}.

RNA blots of a variety of rat tissues and PC12 cells revealed that, unlike the other synaptobrevins, the messenger RNA for the new form of synaptobrevin is not expressed primarily in brain but in all tissues and cells tested (Fig. 2a). To determine if the mRNA was translated in these tissues, an antibody was raised to the unique amino terminus of the new synaptobrevin. Immunoblots of rat tissues confirmed that the new form of synaptobrevin was ubiquitously expressed (Fig. 2b). Because the new form of synaptobrevin appears to be present in all cells, we named the protein cellubrevin as the cellular homologue of synaptobrevins.

The intracellular distribution of cellubrevin was investigated by immunofluorescence and subcellular fractionation experiments. Because endogenous levels of cellubrevin are very low in cultured cells, cells transfected with a cellubrevin expression vector were used for the immunofluorescence experiments. In these cells, cellubrevin is localized to small vesicles present in all parts of the cells but particularly concentrated around the Golgi complex (Fig. 3a). Comparison of the distribution of cellubrevin-positive microvesicles with that of transferrin taken up from the medium or of transferrin receptors reveals that all transferrin-recycling vesicles also stain for cellubrevin (Fig. 3a, and data not shown). Cellubrevin antibodies stain many more vesicles than transferrin or antibodies to its receptor, suggesting that cellubrevin is a component not only of the vesicles recycling transferrin-receptors but also of other similar vesicles.

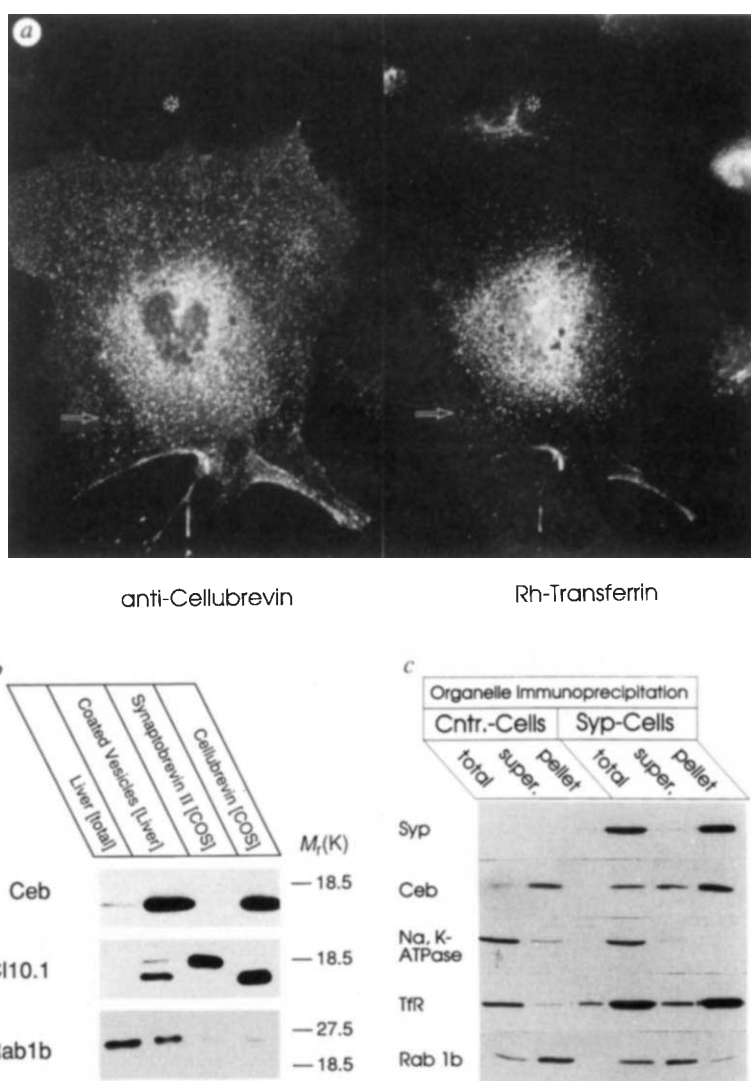
To obtain quantitative evidence for the presence of cellubrevin in the receptor-mediated endocytosis pathway and its recycling organelles, subcellular fractionations were done. The levels of cellubrevin in total rat liver membranes and in purified coated vesicles were determined (Fig. 3b). Cellubrevin was enriched over 100 times in coated vesicles compared to total membranes, suggesting that cellubrevin is a specific component of coated vesicles.

As an independent approach to determining the localization of cellubrevin in cells, a well characterized Chinese hamster ovary (CHO) cell line transfected with synaptophysin was used. In these cells, synaptophysin is colocalized with the transferrin receptor and targeted to the receptor-mediated endocytosis pathway^{13–15}. Synaptophysin-containing organelles were immuno-isolated from synaptophysin-expressing CHO cells and from control cells, and the distribution of endogenous cellubrevin and several marker proteins was analysed in all fractions by immunoblotting (Fig. 3c).

Endogenous cellubrevin in CHO cells quantitatively coisolates with synaptophysin, suggesting that they are colocalized on the same vesicles (Fig. 3c). Transferrin receptors are also coimmunoprecipitated, whereas rab1b and Na, K-ATPase, two proteins that are not enriched in the pathway of recycling receptors,

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FIG. 3 Subcellular localization of cellubrevin. **a**, Immunofluorescence localization of cellubrevin in transfected CV-1 cells. CV-1 cells transfected with a cellubrevin expression vector were fixed after a 1-h incubation with rhodamine-labelled transferrin and immunostained for cellubrevin using fluorescein-labelled secondary antibodies. The asterisk marks the position of a non-transfected neighbouring cell that is not labelled by the cellubrevin antibody but is transferrin positive. For easier orientation, the arrow points to a pair of vesicles identically labelled by transferrin and anti-cellubrevin antibodies. **b**, Enrichment of cellubrevin in rat liver coated vesicles. Triton X-114-extracted rat liver membranes (left lane, corresponding to 100 μ g total membrane protein) and purified coated vesicles (second lane, 18 μ g protein) were analysed by SDS-PAGE and immunoblotting using a cellubrevin-specific antibody (top panel), an antibody that recognizes multiple synaptobrevins including cellubrevin (C110.1, middle panel), and an antibody to rab1B as a negative control (bottom panel). In the right two lanes, COS cells transfected with expression vectors encoding synaptobrevin II (migrating slower than cellubrevin) and cellubrevin were analysed as positive controls. The 18K band observed in liver coated vesicles with C110.1 may represent an unidentified additional form of synaptobrevin. **c**, Copurification of endogenous cellubrevin with synaptophysin and transferrin receptors in transfected CHO cells in which synaptophysin was previously shown to be targeted to the receptor-mediated endocytosis pathway^{13,14}. Immunoblots of three fractions obtained during immuno-isolation of synaptophysin-containing microvesicles from control and from synaptophysin-transfected CHO cells: the starting materials (left lanes), the Triton X-114-extracted supernatants from the immunoprecipitations (middle lanes), and the immunoprecipitates (right lanes). Immunoblots were stained with antibodies to synaptophysin (syp), cellubrevin (ceb), Na, K-ATPase, transferrin receptors (TfR), and rab1b. **METHODS**. Expression vectors for synaptobrevin II and cellubrevin were constructed in pCMV2 and pCMV5, Qiagen-purified and transfected into CV-1 cells (ATCC) using standard procedures^{13,25}. Transfected CV-1 cells were transferred to glass cover slips one day before immunofluorescence staining and incubated with rhodamine-labelled transferrin (25 mg l^{-1} for 1 h at 37 °C in DMEM) before fixation and immunofluorescence staining as described.^{13,14}. Clathrin-coated vesicles were purified from fresh rat liver²⁶. Purity was verified by negative staining electron microscopy (> 90% coated profiles). Subcellular fractionations of CHO cells by immunoprecipitations with antibodies to synaptophysin were done as described^{14,24}. Fractions were analysed by immunoblotting using antibodies to the indicated proteins and enhanced chemiluminescence (Amersham) for



detecting reactive bands. Levels of endogenous cellubrevin were very low in all cells and tissues tested, requiring more than 10 times longer exposure for cellubrevin than for other proteins. For quantifications of protein levels, immunoblots were reacted with iodinated secondary antibodies and the signal was measured in a phosphorimager.

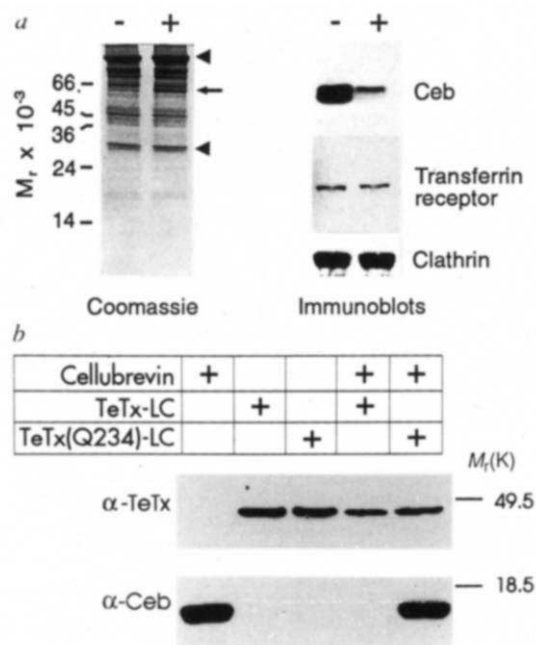


FIG. 4 Cellubrevin as a tetanus toxin target. **a**, Selective cleavage of cellubrevin by tetanus toxin light chain in rat liver coated vesicles. SDS-polyacrylamide gels of coated vesicles incubated in the absence and presence of tetanus toxin light chain were analysed by Coomassie blue staining (left panel; arrow heads indicate positions of clathrin heavy and light chains, and full arrow position of tetanus toxin light chain) and by immunoblotting for cellubrevin, transferrin receptors, and clathrin heavy chain (right panels). **b**, Selective degradation of cellubrevin by wild-type but not by mutant tetanus toxin light chains in transfected cells. COS cells were cotransfected with vectors encoding cellubrevin and wild-type tetanus toxin light chain (TeTx-LC) or tetanus toxin light chain bearing an inactivating point mutation (TeTx(Q234)-LC)⁶. Transfected cells were analysed by immunoblotting using antibodies to tetanus toxin and to cellubrevin, demonstrating selective degradation of cellubrevin if cotransfected with wild-type tetanus toxin. Numbers on the right indicate M_r markers. **METHODS**. Purified rat liver coated vesicles (100 μ g) were incubated with 5 nM recombinant tetanus toxin light chain in 0.1 ml volume at 37 °C for 1 h⁶. Eukaryotic expression vectors encoding wild-type and mutant tetanus toxin light chains were constructed by cloning the *EcoRI*-*HindIII* insert fragments from the bacterial expression vectors⁶ encoding residues 1 to 457 of tetanus toxin^{27,28} into the same sites of pCMV5. Samples from COS cells transfected with the expression vectors²⁷ were analysed by SDS-PAGE followed by immunoblotting using monoclonal antibodies to tetanus toxin and to transferrin receptors and polyclonal antibodies to cellubrevin and clathrin heavy chain. Immunoreactive bands were visualized by enhanced chemiluminescence.

chain also cleaves cellubrevin.

First, purified coated vesicles were incubated with tetanus toxin light chain. There was no change in the protein composition of the coated vesicles. But cellubrevin was almost completely degraded, suggesting that tetanus toxin light chain selectively proteolysed this protein (Fig. 4a). Second, cellubrevin was cotransfected with wild-type tetanus toxin light chain (TeTx-LC) or an inactive mutant light chain (TeTx(Q234)-LC) into COS cells. Only cotransfection of cellubrevin with wild-type tetanus toxin light chain abolished cellubrevin expression (Fig. 4b). Thus cellubrevin is a good substrate for tetanus toxin light chain in subcellular fractions and in cells. Because of the unavailability of assays to measure exocytotic fusion of receptor-carrying vesicles in cells, it remains to be established whether tetanus toxin interferes with this process.

Cellubrevin is ubiquitously distributed in a constitutively recycling vesicular pathway in cells, the receptor-mediated endocytosis pathway. As the target of tetanus toxin action in nerve terminals, synaptobrevin is thought to be involved in synaptic vesicle fusion. Cellubrevin is also a target for tetanus toxin and may also be part of a membrane-fusion complex. This demonstrates that tetanus toxin is active in non-neuronal cells which it cannot usually enter because of the inability of these cells to take up the toxin. Our results indicate that constitutive and regulated membrane trafficking pathways may be mechanistically very similar, possibly differing only in key regulatory steps. They suggest a close evolutionary relationship between these pathways and open new avenues for investigating the functions of the proteins involved in these pathways. □

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Complementation of *byr1* in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase

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INTRACELLULAR signalling from receptor tyrosine kinases in mammalian cells involves the activation of a signal cascade which includes p21^{ras} and the protein kinases p74^{raf-1}, MAP kinase kinase and MAP kinases^{1–8}. In the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* the response to mating pheromones requires the Spk1 and KSS1/FUS3 kinases, which have sequence homology to vertebrate MAP kinases^{9–12}. The recent cloning of complementary DNAs for mammalian^{13–15} and frog¹⁶ MAP kinase kinases has shown that they are homologous to the *S. pombe* Byr1 (ref. 17) and *S. cerevisiae* STE7 (ref. 18) kinases, which have been proposed to function upstream of Spk1 and KSS1/FUS3, respectively^{19–22}. We have investigated whether these apparently similar kinase pathways are functionally conserved between vertebrates and *S. pombe*. We report here that expression of mammalian MAP kinase kinase alone fails to complement a *byr1* mutant of *S. pombe*. When coexpressed with Raf kinase, however, MAP kinase kinase is activated by phosphorylation and the mating defect of the *byr1* mutant is rescued. This suggests that the pathways are functionally homologous and that Raf kinase may directly phosphorylate and activate MAP kinase kinase.

Mammalian MAP kinase kinase (MAPKK) is 55% identical in amino-acid sequence to *S. pombe* Byr1 in the catalytic domain and 38% identical overall. This suggested that MAPKK might

be able to complement the *byr1* mutant defect. To test this, a rabbit MAPKK cDNA driven by a fission yeast promoter was transformed into a *byr1* mutant strain. Expression of MAPKK could not complement the mating defect of this strain (Fig. 1). Because MAPKK must be activated by phosphorylation²³ it was possible that *S. pombe* did not have a kinase that could activate mammalian MAPKK. The product of the *raf-1* proto-oncogene has been implicated in activation of the MAP kinase pathway in mammalian cells, perhaps as a direct activator of MAPKK^{6,8}, so we examined whether Raf-1 could activate MAPKK in *S. pombe*. When *byr1* mutant cells coexpressed either Raf-1 or Δ Raf-1, an activated derivative of Raf-1 (ref. 7), together with MAPKK they were able to mate (Fig. 1), although the mating frequency was lower than that of cells carrying the wild-type *byr1*⁺ gene (Table 1). Expression of Raf-1 or Δ Raf-1 alone or MAPKK alone did not allow *byr1* mutant cells to mate, showing that expression of both MAPKK and Raf is required to substitute for Byr1. Furthermore, functional kinase activity of MAPKK was required because a kinase-deficient mutant of MAPKK (Lys 97 to Met) coexpressed with Raf failed to complement *byr1* (data not shown).

Rescue of the *byr1* deficiency by MAPKK plus Raf suggests that elements regulated by Byr1 kinase in *S. pombe* are responsive to mammalian MAPKK. One such element may be the Spk1 protein kinase, which is homologous to vertebrate MAP kinases and to *S. cerevisiae* FUS3 and KSS1 and, like them, contains the regulatory TEY phosphorylation site in subdomain VIII⁹. Both mammalian and *Xenopus* MAP kinase can partially substitute for Spk1 in *S. pombe*^{19,20}. From genetic and biochemical analysis^{19,20} and by analogy to other systems^{22,24}, it is probable that Byr1 phosphorylates and activates Spk1. Activated MAPKK may thus be substituting for Byr1 by phosphorylating and activating the Spk1 kinase in *S. pombe*. Consistent with this hypothesis, coexpression of MAPKK and Raf could not rescue the mating deficiency of a *spk1* null mutant (Table 1).

These experiments show that mammalian MAPKK required

coexpression of Raf to function in *S. pombe*. To investigate whether the kinase activity of MAPKK was dependent on Raf, cell extracts were prepared from *S. pombe* cells expressing MAPKK alone, MAPKK plus Δ Raf-1 or Δ Raf-1 alone, fractionated on a Mono-Q ion-exchange column, and the fractions assayed for MAP kinase kinase activity or MAP kinase activity (Fig. 2). MAPKK activity was detectable only in cells coexpressing MAPKK plus Δ Raf-1. Immunoblot analysis of total cell extracts showed that expression of Δ Raf-1 did not affect the level of expression of MAPKK (data not shown). The elution pattern of active MAPKK from the Mono-Q column was complex, with four peaks of activity that correlated well with the position of MAPKK in immunoblots (Fig. 2b). In cells expressing Δ Raf-1, the peak of MAPKK immunoreactivity eluted at ~ 100 mM NaCl (fraction 19), which corresponded to the most active fraction and is similar to the position of the major peak of MAPKK activity from mammalian cells²⁵. In the absence of Raf, most of the MAPKK was found in the column flow-through fractions (Fig. 2b). No MAP kinase activity could be detected in any of these extracts.

The elution pattern of active MAPKK suggested some modification of the protein in cells expressing Raf. Because Raf-1 is a protein kinase we looked at the phosphorylation state of MAPKK in *S. pombe* after metabolic labelling with [³²P]orthophosphate. Although MAPKK was phosphorylated in the absence of Raf-1, coexpression of Raf-1 or Δ Raf-1 led to hyperphosphorylation of MAPKK which was accompanied by a decrease in its mobility upon gel electrophoresis (Figs 2 and 3). We estimated that Raf-1 stimulated MAPKK phosphorylation about fourfold and that Δ Raf-1 gave at least a fivefold stimulation. In Raf-1-expressing cells, two forms of MAPKK were present in similar amounts, whereas in Δ Raf-1-expressing cells the slower migrating form predominated (Fig. 3). Given that Δ Raf-1 is more effective than Raf-1 in activating MAPKK as judged by complementation of *byr1* (Table 1), the slower-migrating hyperphosphorylated form of MAPKK is likely to be the biochemically active form. Phosphoamino-acid analyses showed that hyperphosphorylated MAPKK contained phosphoserine and phosphothreonine but no detectable phosphotyrosine (data not shown), in agreement with studies on active MAPKK from *Xenopus* oocytes²⁶.

TABLE 1 Mating frequency (%) of *byr1*, *byr2* and *spk1* mutants transformed with mammalian kinase genes

Plasmids	Mutant strains		
	<i>byr1</i> Δ (CB53)	<i>byr2</i> ⁻ (CB59)	<i>spk1</i> Δ (CB57)
MAPKK	0 (1362)	0 (2177)	0 (747)
MAPKK + raf-1	1.36 (736)	ND	0 (854)
MAPKK + Δ raf-1	3.30 (1755)	4.30 (2045)	0 (1050)
raf-1	0 (1574)	ND	ND
Δ raf-1	0 (1538)	0 (2760)	ND
MAPKK + <i>byr2</i> ⁺	0 (1708)	ND	ND
<i>byr1</i> ⁺	52.0 (477)	ND	ND
<i>byr2</i>	ND	25.1 (742)	ND
<i>spk1</i> ⁺	ND	ND	39.0 (267)

Cells were grown on sporulation agar (SSA) at 30 °C for 4 days and the number of zygotes, asci and unmated cells were counted. Two clones for each transformant were examined and the average mating frequency determined. Numbers in parentheses are the total number of cells counted for each transformant. The full genotypes of the mutant strains are CB53: *h*⁹⁰ *byr1::ura4* Δ RS *ade6 leu1 ura4*; CB59: *h*⁹⁰ *byr2-JM86 ade6 leu1 ura4*, and CB57: *h*⁹⁰ *spk1::ura4* Δ RS *ade6 leu1 ura4*, which was derived from the *spk1* mutant described in ref. 9. The plasmids are described in the legend to Fig. 1, except for the *byr2*⁺ plasmid which has the *byr2*⁺ gene cloned into pREP42 and the *spk1*⁺ plasmid which is from ref. 9. ND, Not determined.

The hyperphosphorylation of MAPKK in cells expressing Raf could be the result of phosphorylation on new sites, enhanced phosphorylation on the sites phosphorylated in the absence of Raf, or a combination of both mechanisms. To investigate MAPKK phosphorylation in more detail, tryptic phosphopeptide maps of immunoprecipitated MAPKK were generated (Fig. 3). The maps from Raf-1 and Δ Raf-1-expressing cells were identical but distinct from the map from cells expressing MAPKK alone (Fig. 3). The most heavily labelled phosphopeptide is peptide a in cells expressing Δ Raf-1 but peptide b in cells without Raf. Phosphopeptide c is only seen in cells expressing Raf kinase. MAPKK phosphorylation in the absence of Raf may be the result of autophosphorylation, which occurs *in vitro*¹⁴, or of

FIG. 1 Complementation of the mating defect of a *byr1* mutant by coexpression of MAPKK and Raf. Micrographs of a *byr1* mutant transformed with either MAPKK alone (a), MAPKK plus Raf-1 (b), MAPKK plus Δ Raf-1 (c), Raf-1 alone (d), Δ Raf-1 alone (e) and *S. pombe byr1*⁺ (f).

METHODS. Rabbit MAPKK cDNA and *S. pombe byr1*⁺ were cloned into pREP41 and human *raf-1* and *Δraf-1* were cloned into pREP42. The vector plasmids use the *S. pombe nmt1* promoter and carry either the *S. cerevisiae LEU2* gene (pREP41) or the *S. pombe ura4*⁺ gene (pREP42) as selectable markers³¹. A null mutant of *byr1*, with a 0.18-kilobase deletion (S_{pel} to B_{am}HI) of the open reading frame, was constructed by one-step gene disruption. The *byr1* mutant strain CB53 (*h*⁹⁰ *byr1::ura4* Δ RS *ade6 leu1 ura4*) was transformed with two plasmids, one derived from pREP41 and the other from pREP42, and Leu⁺ Ura⁺ transformants carrying both plasmids were selected. Transformants were grown on synthetic sporulation agar (SSA)³² for 3 days and then photographed. Molecular genetic methods for *S. pombe* were as described^{33,34}.

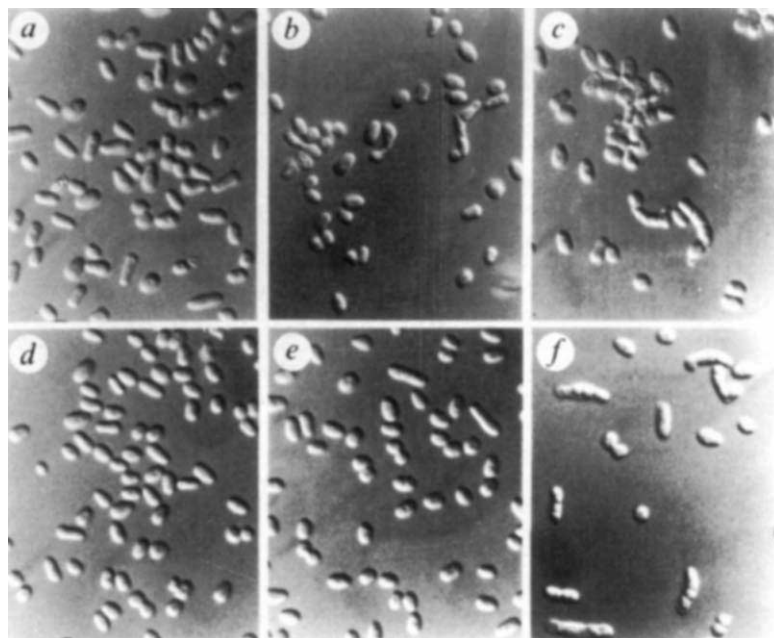


FIG. 2 MAPKK activation in *S. pombe* cells coexpressing Raf. **a**, MAPKK and MAPK activities of fractionated cell extracts from a *byr1* mutant strain (CB53) transformed with MAPKK and Δ raf-1. **b**, Immunoblot of column fractions to detect rabbit MAPKK. **METHODS**. **a**, Cells were grown in minimal medium (EMM) to $\sim 1 \times 10^7$ cells ml^{-1} , collected by centrifugation and washed in stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 , pH 8.0). Cells (2.0×10^6) suspended in 20 μl 50 mM Tris-Cl pH 7.3, 40 mM $\text{Na}_2\text{P}_2\text{O}_7$, 50 mM NaF, 5 mM MgCl_2 , 0.3 mM sodium orthovanadate, 10 mM EGTA, 1% Triton X100, 20 $\mu\text{g ml}^{-1}$ leupeptin, 20 $\mu\text{g ml}^{-1}$ aprotinin, 1 mM PMSF were broken with 1 g glass beads by vortexing for 2 min, the beads washed with 50 volumes of buffer A (50 mM Tris-Cl pH 7.0, 2 mM EDTA, 2 mM EGTA, 0.1% β -mercaptoethanol, 5% glycerol, 0.03% Brij35, 0.3 mM sodium orthovanadate, 1 mM benzamide, 4 $\mu\text{g ml}^{-1}$ leupeptin) and the lysate cleared by centrifugation in an Eppendorf microfuge at 14,000 r.p.m. for 10 min. 0.5 ml cleared lysate (1.5 mg total protein) was then applied to a 1 ml Mono-Q column (Pharmacia LKB) and the column developed in buffer A with a 25-ml linear salt gradient to 0.35 M NaCl. The flow rate was 1 ml min^{-1} and 0.5-ml fractions were collected and assayed for myelin basic protein (MBP) kinase activity after preincubation with recombinant ERK2 (+ERK2) for MAPKK activity or after preincubation with buffer (-ERK2) for MAPK activity, essentially as described in ref. 25. **b**, Aliquots of each fraction assayed for kinase activity were resolved by 10% SDS-PAGE, electroblotted to Immobilon (Millipore) and probed with rabbit polyclonal antibody 179 raised against a GST-rabbit MAPKK fusion protein (A.A. and C.J.M., unpublished results) and ECL reagents (Amersham).

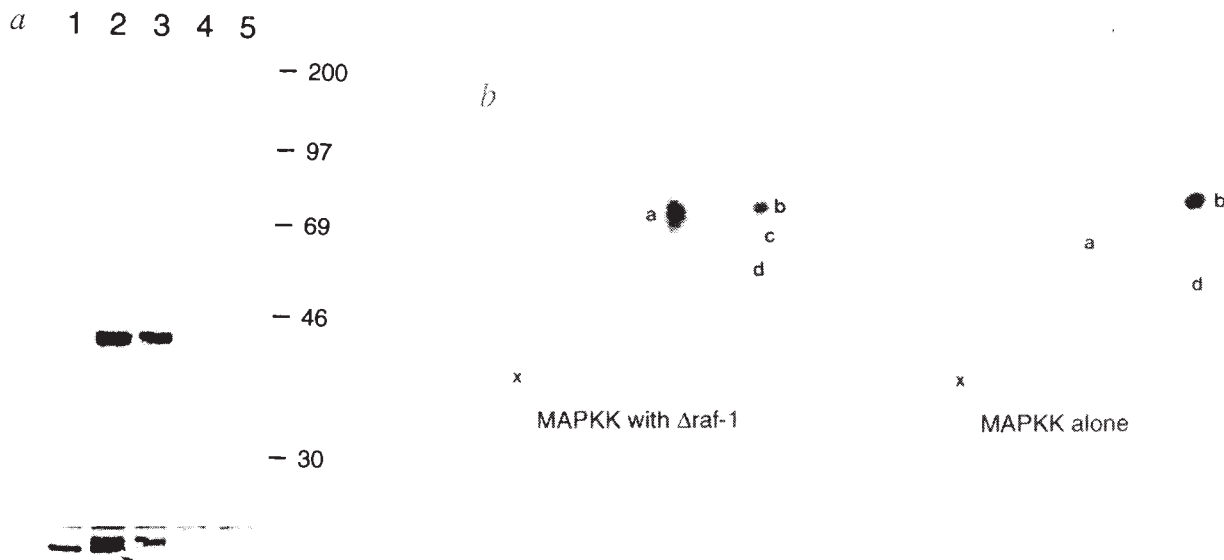
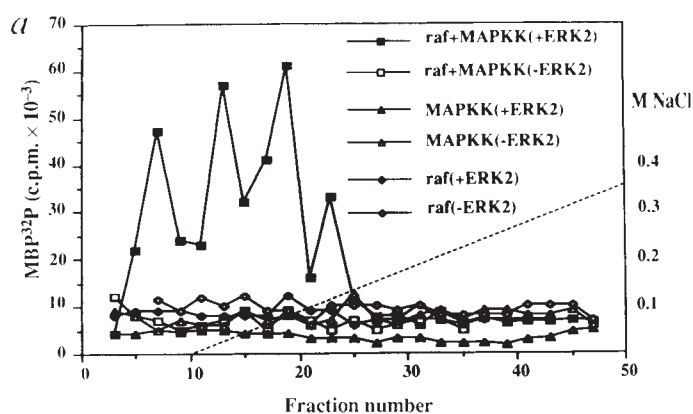


FIG. 3 Stimulation of MAPKK phosphorylation by Raf in *S. pombe*. **a**, Immunoprecipitation of rabbit MAPKK in cells expressing MAPKK alone (lane 1), MAPKK plus Raf-1 (lane 2), MAPKK plus Δ Raf-1 (lane 3), Raf-1 alone (lane 4) and Δ Raf-1 alone (lane 5). Top, Phosphorimager print showing phosphate-labelled MAPKK (lanes 1-3). Bottom, Immunoblot of the same immunoprecipitated samples with the anti-MAPKK serum. **b**, Phosphopeptide maps of MAPKK. The origin is marked with a cross (bottom left of each panel). The horizontal dimension is electrophoresis (cathode to right) and the vertical dimension is chromatography. **METHODS**. Cells were grown in low-phosphate EMM to mid-log phase ($\sim 5 \times 10^6$ cells ml^{-1}), labelled with [^{32}P]orthophosphate for 3.5 h (ref. 33) and then broken with glass beads in lysis buffer (25 mM Tris-Cl, pH 8.0, 40 mM $\text{Na}_2\text{P}_2\text{O}_7$, 50 mM NaF, 5 mM MgCl_2 , 0.1 mM sodium orthovanadate, 10 mM EGTA, 1% Triton X100, protease inhibitors: 20 $\mu\text{g ml}^{-1}$ aprotinin, 20 $\mu\text{g ml}^{-1}$ leupeptin, 1 mM PMSF). After centrifugation (15,000 r.p.m. for 15 min) the supernatants were adjusted to 0.5% sodium deoxycholate (Na-DCC), 0.1% sodium dodecyl sulphate

(SDS), and incubated for 1 h with anti-MAPKK serum 179 precoupled to protein A-Sepharose. The beads were washed 4 times with wash buffer (lysis buffer with Na-DCC and SDS, without MgCl_2 and protease inhibitors), incubated with 0.1 mg ml^{-1} RNase for 30 min, washed again, suspended in Laemmli's sample buffer and boiled. Samples were resolved by 10% SDS-PAGE and electroblotted to Immobilon. After autoradiography, MAPKK was detected using the anti-MAPKK serum 179 and alkaline-phosphatase-conjugated secondary antibody (Promega). Radioactivity was detected using a phosphorimager (Molecular Dynamics) and the immunoblot was scanned on a scanning densitometer (Joyce-Loebl). Two-dimensional phosphopeptide maps were obtained after trypsin digestion of MAPKK on Immobilon. The first dimension was electrophoresis in pH 1.9 buffer at 400 V for 60 min on cellulose thin-layer plates (Kodak); the second dimension was ascending chromatography developed with phosphochromatography buffer for 3 h (ref. 35).

phosphorylation by an endogenous yeast kinase. Whatever the cause, this Raf-independent phosphorylation does not activate the enzyme.

Immunoprecipitates of Raf kinase from mammalian cells phosphorylate and reactivate phosphatase-treated homogenously pure MAPKK preparations⁷ and bacterially expressed v-Raf can also reactivate partially purified MAPKK⁸; but these experiments do not rule out an intermediate between Raf and MAPKK²⁷. The inability of *S. pombe* to activate MAPKK unless Raf is expressed strongly suggests that Raf directly phosphorylates and activates MAPKK. We observe that coexpression of Raf and MAPKK, but not Raf alone, also suppresses the sterility of a mutant defective in *byr2* (Table 1) which encodes a protein kinase thought to function upstream of Byr1^{28,29}. The inability of Raf alone to suppress a mutant that contains an intact *byr1* gene shows that Raf cannot activate Byr1. This provides a strong genetic argument that Raf directly phosphorylates and activates MAPKK because a putative intermediate would have to be able to activate mammalian MAPKK but not Byr1, its *S. pombe* homologue.

Recently a putative MAPKK kinase (MEK kinase)³⁰ distinct from Raf has been identified in mammalian cells which shares considerable sequence similarity with Byr2 kinase and its *S. cerevisiae* homologue, STE11. Although overexpression of MEK kinase can activate MAPKK in mammalian cells, Table 1 shows that overexpression of Byr2 does not activate MAPKK in *S. pombe*, arguing that the regulation of MAPKK has diverged from that of Byr1.

Finally, it may be possible to exploit the reconstituted MAP kinase activation pathway described here to identify further components of the pathway and as an *in vivo* screen for agents that can inhibit the mammalian kinases kinases. □

Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*

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THE Ras proteins are key regulators of the growth of eukaryotic cells, but their direct target enzymes, or 'effectors', are unknown¹. The protein encoded by the *c-raf-1* proto-oncogene is thought to function downstream of p21^{as} because disruption of Raf blocks signalling by Ras in a number of systems²⁻⁵. Here we report that the amino-terminal cysteine-rich regulatory region of p74^{c-raf-1} expressed as a glutathione-S-transferase (GST) fusion protein binds directly to Ras with relatively high affinity (50 nM). The binding is strictly dependent on the Ras protein being in the active GTP-bound conformation rather than the inactive GDP-bound state. Raf-GST interacts with wild-type and oncogenic Ras (Val 12) but fails to interact with a biologically inert effector mutant of Ras (Ala 38) and a dominant negative mutant (Asn 17). A peptide based on the effector region of Ras inhibits the interaction. Raf-GST acts as a potent competitive inhibitor of the GTPase-activating proteins p12^{GAP} and neurofibromin. In addition, Raf itself displays weak GTPase-stimulating activity towards Ras. It is therefore likely that Raf is a direct effector of Ras.

The N-terminal region of the *c-raf-1* protein contains a characteristic pattern of cysteine residues thought to be involved in binding to zinc ions, and related to regulatory domains of a number of enzymes, most notably those of the protein kinase C family in which they serve as diacylglycerol binding sites⁶. This region of Raf-1 when expressed by itself in fibroblasts inhibits the function of normal, but not oncogenic, Raf proteins: it has therefore been proposed to act as the site of interaction of Raf with an incoming activating signal, which could be either a pro-

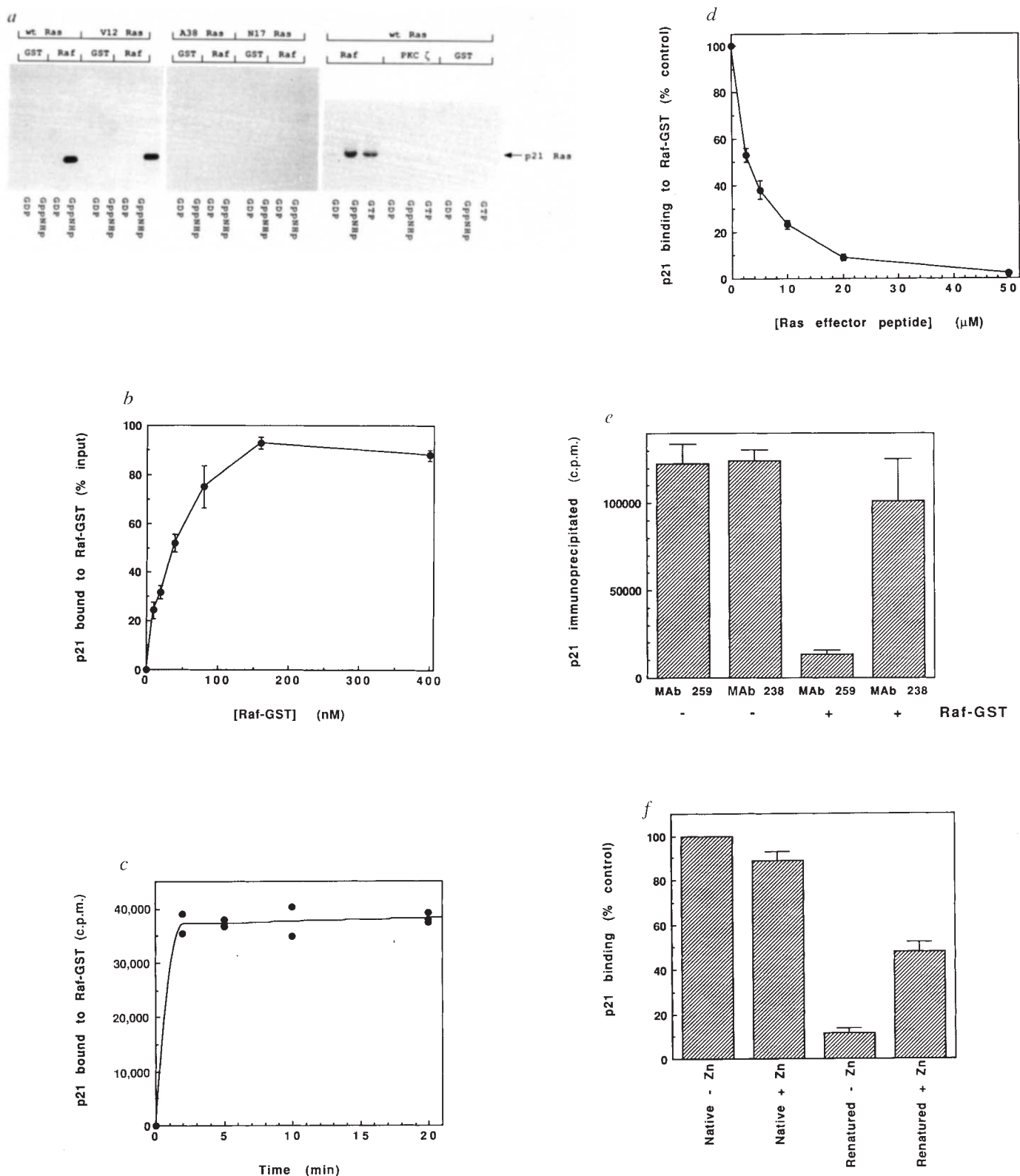
FIG. 1 Direct binding of GTP-bound Ras to the N-terminal region of Raf. a, Ras proteins were loaded with GTP, GppNhp or GDP and mixed with Raf-GST, PKC ζ -GST or GST alone. Complexes were recovered using glutathione agarose, washed and probed for the presence of Ras on immunoblots. b, c-Ha-Ras protein was labelled with [α -³²P]GTP, bound to increasing concentrations of Raf-GST and recovered using glutathione agarose. c, Time course of [α -³²P]GTP-labelled c-Ha-Ras protein binding to Raf-GST. d, Inhibition of [α -³²P]GTP-labelled c-Ha-Ras protein binding to Raf-GST by an effector peptide from Ras (residues 17-44). e, Raf-GST interaction with Ras interferes with the binding of monoclonal antibody Y13-259 to Ras. f, Renaturation of Raf-GST in the presence or absence of zinc: effect on binding to Ras.

METHODS. a, Each assay contained 50 pmol purified bacterially expressed Ras protein which was greater than 50% active by nucleotide binding. Nucleotide loading was done in 50 mM HEPES, pH 7.5, 5 mM EDTA, 5 mg ml⁻¹ bovine serum albumin, 500 μ M guanine nucleotide for 3 min at 30 °C, before addition of 10 mM MgCl₂ and cooling to 0 °C. Binding reactions were done in 50 μ l of 50 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 20 μ M ZnCl₂, 1 mg ml⁻¹ BSA with 0.5 μ g GST fusion protein for 2 h at 4 °C. Glutathione agarose (10 μ l) was added to each incubation for a further 1 h to retrieve the GST fusion proteins, which were then washed with 4 $\times$ 1 ml of ice cold 50 mM HEPES pH 7.5, 100 mM KCl, 5 mM MgCl₂, 20 μ M ZnCl₂, 0.1% Triton X100. The washed beads were subjected to SDS-PAGE on a 12.5% gel and immunoblotted for Ras using pan-Ras 11 monoclonal antibody (DuPont). GST fusion proteins were made using the pGEX-2T vector (Pharmacia). The N-terminal regulatory domains of c-Raf-1 (residues 1-257) and PKC ζ (residues 1-250) were obtained using the polymerase

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chain reaction from a human placental cDNA library (Raf) or cloned DNA (PKC ζ). The fusion proteins were purified following the manufacturer's protocol with the addition of a Superdex-75 FPLC sizing column step. *b*, *c*-Ha-Ras protein (0.25 pmol, 10 nM final concentration in assay) was loaded with 0.5 μ Ci of [α - 32 P]GTP as in *a*. Unlabelled GDP (0.1 mM) was included after labelling was complete. Binding to Raf-GST and glutathione agarose was as above in 25 μ l with the omission of ZnCl $_2$. After washing the glutathione-agarose was counted for 32 P label. Results are mean of triplicates and are expressed as per cent of labelled Ras put into assay as determined using anti-Ras antibody Y13-259. *c*, Assays

done as in *b* except that 0.5 μ g Raf-GST per assay was precoupled to glutathione agarose before adding to Ras. *d*, Assays as in *b* using 0.05 μ g Raf-GST. *e*, Assays as in *c*, except Ras was immunoprecipitated at end for 1 h with antibody 259 or 238 precoupled to rabbit anti-rat/protein A sepharose. *f*, Raf-GST was denatured in urea/EDTA and renatured in the presence or absence of 100 μ M zinc as described in ref. 13. 0.5 μ g of these preparations were prebound to glutathione-agarose, washed in binding buffer and incubated with labelled Ras as in *c*. Counts bound are expressed as per cent of those seen with non-denatured Raf. The means of triplicates are shown with standard errors.

tein or a lipid^{2,3}. The N-terminal region of Raf also disrupts signalling from both normal and transforming mutants of Ras, suggesting that it is sequestering a signal generated by activated Ras³. We therefore expressed this part of *c-raf-1* (amino acids 1–257) as a glutathione-S-transferase fusion protein in bacteria in order to investigate the nature of the stimulatory moiety that binds to Raf at this site. As a first step the possibility was investigated that Ras could interact directly with Raf. Purified bacter-

ially expressed c-Ha-Ras protein was loaded with either GDP, GTP or a non-hydrolysable analogue of GTP, GppNHp, and mixed with the Raf-GST fusion protein. The fusion protein was then bound to glutathione agarose, washed and the presence of Ras investigated by immunoblotting. Ras binds strongly to Raf-GST, but only when loaded with GTP or a GTP analogue and not when GDP-bound (Fig. 1a). As controls, Ras fails to bind to either GST itself or to a GST fusion protein containing the cysteine motif (residues 1–250) from the related enzyme, protein kinase C ζ (ref. 7). In addition to wild-type Ras, the oncogenic mutant Val 12 binds tightly to Raf-GST when GTP bound. An effector mutant of Ras, Ala 38, which is biologically inactive and thought to be incapable of interacting with the key targets of Ras, is unable to bind to Raf-GST in this assay. In addition, a dominant negative mutant of Ras, Asn 17, fails to bind Raf even when GTP bound. Similar observations have been made previously for adenyl cyclase, a yeast effector of Ras, which is not activated by GTP-bound Asn-17 Ras. This has been interpreted as indicating that this mutant fails to adopt an activated conformation in response to GTP binding⁸.

The interaction of GTP-bound Ras with Raf-GST occurs with high affinity (Fig. 1b). The affinity of the interaction is about 50 nM: this is somewhat stronger than that for interaction of Ras and neurofibromin (250 nM) and much stronger than for the Ras and p120^{GAP} interaction (5 μ M)⁹. It is not known whether the interaction of Ras and full-length Raf will be of similarly high affinity; the inability so far to detect Raf in immunoprecipitates of Ras from mammalian cells suggests that it may not be, or that it is transient because of modifications occurring in the catalytic region of Raf. The interaction is also extremely rapid, going to completion at 0 °C in just a few minutes (Fig. 1c).

Because Ala-38 Ras fails to bind Raf-GST, it is likely that the interaction occurs at the effector site of Ras; the ability of a synthetic peptide based on the sequence of Ras in this region (amino acids 17–44) to inhibit this binding was therefore tested. The peptide inhibits Ras binding to Raf-GST with a half-maximal inhibitory concentration (IC₅₀) of about 3 μ M (Fig. 1d): this is similar to the ability of this peptide to inhibit interaction of Ras with p120^{GAP} (ref. 10). Small molecules that block the interaction of Ras with Raf might be capable of inhibiting the growth of Ras-transformed cells¹¹. The involvement of the effector region of Ras in the binding of Raf-GST is further implicated by the observation that monoclonal antibody Y13-259, which blocks the biological activity of Ras when microin-

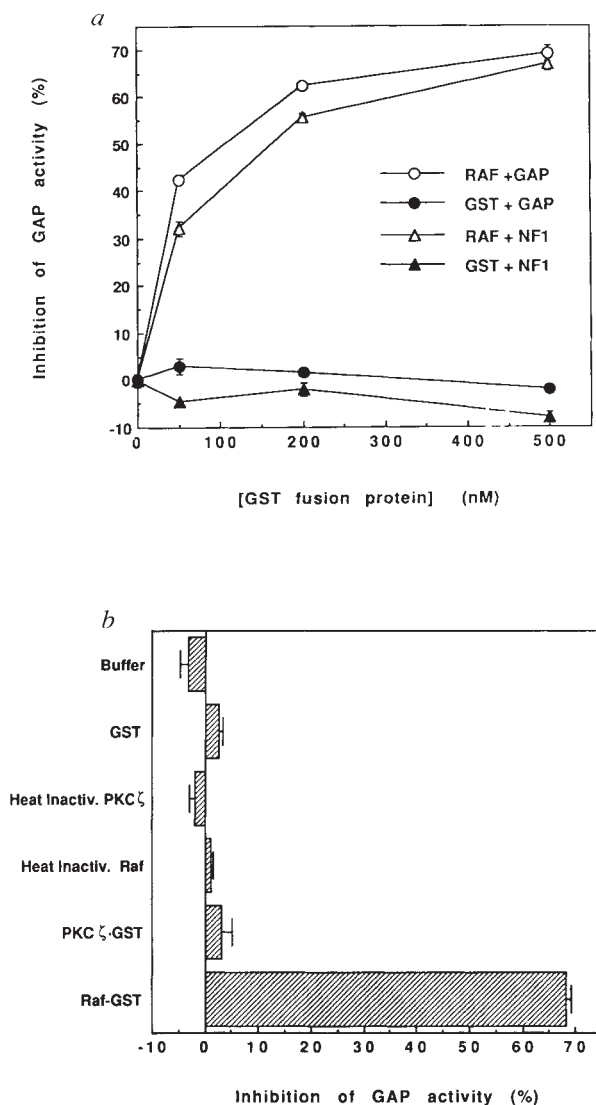


FIG. 2 Inhibition of the Ras-GAP activity of p120^{GAP} and neurofibromin by Raf. **a**, The effect of increasing concentrations of Raf-GST and GST alone on the stimulation of GTP hydrolysis on Ha-Ras by p120^{GAP} and neurofibromin is shown. Percentage inhibition of GAP activity is plotted against GST fusion protein concentration. **b**, Ability of 200 nM Raf-GST, heat-inactivated Raf-GST and PKC ζ -GST to inhibit p120^{GAP} activity. **METHODS**. c-Ha-Ras protein (0.1 pmol) was used per point. Ras was loaded with nucleotide as described in Fig. 1 except that 1 μ Ci of [α -³²P]GTP per point was used instead of unlabelled nucleotide. GAP assays were done in 50 μ l of 50 mM HEPES, pH 7.5, 5 mM MgCl₂, 0.1 mg ml⁻¹ BSA, 20 μ M ZnCl₂, 0.1 mM unlabelled GDP at 30 °C for 15 min before stopping by addition of 300 μ l of 50 mM HEPES pH 7.5, 5 mM MgCl₂, 500 mM NaCl, 0.1% Triton X100, 0.005% SDS. Ras was immunoprecipitated with Y13-238 and bound guanine nucleotides were analysed on TLC as described in ref. 18. p120^{GAP} (50 nM, purified from baculovirus expression system) or 20 nM neurofibromin GAP related domain (bacterially expressed) were used. Heat inactivation of Raf-GST was for 5 min at 70 °C.

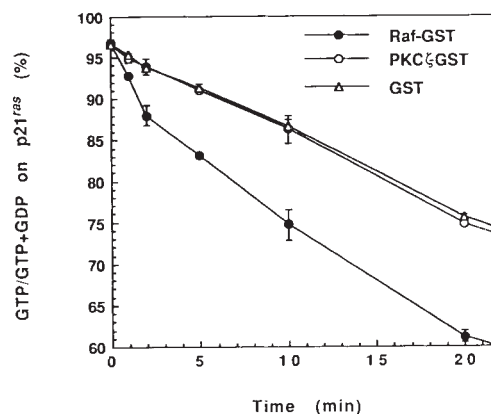


FIG. 3 Ras-GAP activity of Raf-GST. The rate of hydrolysis of GTP bound to Ras is plotted against time at 30 °C in the presence of Raf-GST, PKC ζ -GST or GST alone. GAP assays were as in Fig. 2. GST fusion proteins (200 nM) were used.

jected into cells¹², will not recognize the Ras/Raf-GST complex, whereas Y13-238, which does not inhibit Ras function, does bind the complex (Fig. 1e).

The involvement of the highly conserved cysteine-rich region of Raf in the interaction with Ras was investigated by denaturing Raf-GST in the presence of a chelator and then renaturing in the presence or absence of zinc ions¹³. As shown in Fig. 1f, the presence of zinc increased the ability of renatured Raf-GST to bind Ras. It is therefore likely that the conserved cysteine motif in Raf is involved in binding zinc ions and is important for the interaction with Ras.

The ability of Raf-GST to interfere with the interaction of Ras with other proteins that bind at the same site was investigated. Raf-GST inhibits the ability of the GTPase-activating proteins p12^{GAP} and neurofibromin¹⁴ to stimulate the hydrolysis of GTP on Ras (Fig. 2). Because Raf-GST does not appear to interact with these GAPs directly (data not shown), it is likely that Raf competes with them for access to the effector region of GTP-bound Ras. From the Raf concentration dependence of the inhibition it can be concluded that the binding affinity for the Ras/Raf-GST interaction is about 50 nM, agreeing with that measured by direct binding.

It is noticeable that Raf-GST is unable to inhibit completely the ability of GAPs to stimulate GTP hydrolysis on Ras even at very high concentrations. This suggested that Raf itself could have some GAP activity towards Ras. This possibility was tested directly: as shown in Fig. 3, Raf-GST does stimulate the rate of hydrolysis of GTP on Ras by about threefold, whereas GST and PKC ζ -GST are without activity. This low level of GAP activity compares with maximal stimulations of over 1,000-fold for p12^{GAP} and about 40-fold for neurofibromin⁹. Although the stimulation of GTP hydrolysis on Ras by Raf-GST is weak, it points to a qualitative similarity between the interactions of Ras and Raf and the interaction of Ras and neurofibromin or p12^{GAP} which may have important structural implications. It is not known whether this weak GAP activity reflects a higher level of activity in full-length Raf, although no such activity has yet been observed. Effectors with GAP activity are known in other systems, for example the heterotrimeric G protein G_q and phospholipase C β_1 (ref. 15).

The direct interaction of GTP-bound Ras and Raf-GST *in vitro* is a strong indication that Raf is a direct effector of Ras. Final proof of this must await evidence that Ras can control the kinase activity of full-length Raf, and hence the entire signalling cascade leading to MAP kinase phosphorylation and beyond¹⁶. The question of whether non-oncogenic Ras-related proteins with identical effector regions such as R-Ras and Rap (ref. 17) can interact with Raf kinase will also be of interest. The ability of Raf to interact with Ras directly does not rule out the existence of other effectors of Ras, whether known interacting proteins such as GAP or neurofibromin or others as yet uncharacterized. But the known potency of *v-ras* as a transforming oncogene makes it likely that Raf is responsible for a major part of the effects of Ras on cellular growth. □

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The role of turns in the structure of an α -helical protein

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THE turns joining segments of secondary structure have been proposed to be key elements in dictating the folded structures of native proteins^{1–9}. An alternative view assumes that turns play a passive role and are merely default structures that occur as a consequence of interactions between antiparallel segments of secondary structure, with chain reversal being dictated by the context surrounding the turn and not by the sequence of the turn itself^{10,11}. The solvent-exposure of turns and their tolerance to evolutionary variance suggests that they may have little or no effect on the formation of native structures. Previous investigations have focused on various types of β -turns that connect antiparallel β -strands^{1–3,12,13}, with comparatively little reported on the structural role of interhelical turns. Here we probe the structural importance of such a turn in an antiparallel 4-helix bundle by randomly substituting an interhelical tripeptide in cytochrome b-562 with many different amino-acid sequences. Thirty-one of the resulting substituted proteins were characterized and all of them were shown to fold into stable, native-like structures. These results suggest that this interhelical turn does not play a dominant role in determining the folded structure of this antiparallel 4-helix bundle.

Cytochrome b-562 is a small (M_r 12,000) haem protein found in the periplasm of *Escherichia coli*. In the crystal structure (1.4 Å at $R=0.164$) of this four-helix bundle^{14,15}, the third and fourth helices are joined by the tripeptide Glu₈₁-Gly₈₂-Lys₈₃ (Fig. 1). By substituting this interhelical turn with randomly generated tripeptides, it is possible to construct 8,000 (20^3) possible turn sequences. Among these sequences, those that can form turns compatible with the correctly folded structure can be distinguished by a simple colour assay: cells expressing mutants that fold correctly will bind haem and yield bright red periplasmic extracts (Fig. 2), whereas cells expressing mutant proteins with turns incompatible with a native-like structure will fail to bind haem and yield colourless extracts.

As the Glu₈₁-Gly₈₂-Lys₈₃ turn sequence is located distal to the haem site, we would expect haem binding to be affected only if local perturbations in the turn disrupt the overall native structure. If the sequence of the wild-type turn is essential in directing the 4-helix bundle into its native structure, most of the 8,000 possible sequences will prevent proper folding and produce colourless extracts. If, however, local interactions in the turn do not exert a dominant influence on the structure of the protein (that is, if the turn is passive) then most, perhaps all, of the substitutions should yield bright red samples.

We constructed a library of cytochrome mutants using cassette replacement mutagenesis^{16–18} to randomize codons 81–83. The resulting library encodes all possible tripeptide turn sequences. From this library, 45 independent clones were characterized by DNA sequence analysis and by the red/white

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colour assay (Table 1). Of these, 9 contain termination codons and 31 code for mutant tripeptide sequences (the remaining 5 contain deletions). Of those mutants containing alternative tripeptides in place of the wild-type turn, all 31 sequences give rise to red extracts (Table 1; Fig. 2). The only members of the collection that yield colourless extracts are those encoding nonsense or deletion mutants. These results demonstrate that all the randomly generated tripeptide turn sequences shown in Table 1 are compatible with a stable native-like structure capable of binding haem.

To extend the results of our visual colour assay, mutant proteins with a variety of different turn sequences were purified and characterized. The secondary structures of these proteins were probed by circular dichroism (CD) spectroscopy¹⁹. Figure 3*a* shows that the CD spectrum of a mutant protein (Pro₈₁-Val₈₂-Ala₈₃) is superimposable, within experimental error, upon the

spectrum of the wild-type protein (Glu₈₁-Gly₈₂-Lys₈₃). Similar results were obtained for proteins with the turn sequences Ile₈₁-Asp₈₂-Leu₈₃, Tyr₈₁-Lys₈₂-Leu₈₃, and Ser₈₁-Leu₈₂-Ser₈₃ (data not shown). Calculations of the per cent helix (using PROSEC software; Aviv) for each of these proteins indicates a helicity within 3% of the value for the wild-type protein.

The structures of the mutant and wild-type proteins were also compared using absorption spectroscopy to probe the tertiary structure of the haem binding site. As the absorption spectrum of haem is extremely sensitive to its electronic environment, the spectrum of cytochrome b-562 tests whether varying the interhelical turn disrupts the overall protein structure^{20,21}. As the spectrum of cytochrome b-562 is affected by the oxidation state of the iron²¹, spectra were measured for both the oxidized and reduced forms; both forms of the Ser₈₁-Leu₈₂-Ser₈₃ mutant are compared to those of the wild-type protein in Fig. 3*b* and *c*.

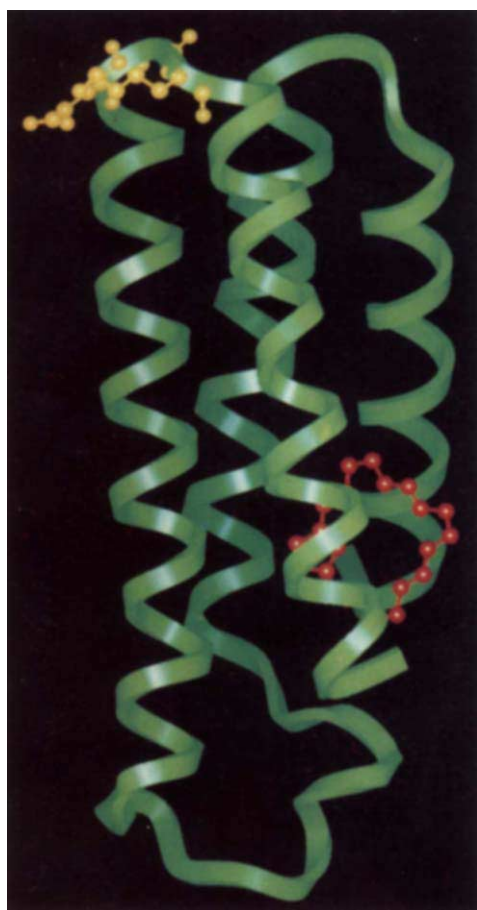


FIG. 1 Ribbon diagram of cytochrome b-562. The haem is shown in red; the sequence Glu₈₁-Gly₈₂-Lys₈₃, which forms an interhelical turn connecting α -helices 3 and 4, is shown in yellow. The ϕ/ψ angles for these residues are $-93.7/8.4$, $78.6/9.9$ and $-93.0/70.9$ for residues 81, 82 and 83 respectively. Coordinates are taken from file 256b in the Brookhaven Data bank³⁴.

METHODS. A 53-mer having the sequence 5'-GCGGGCGGAAGCTTGC-AAATNNNNNNNNGTAAAGAAGCGCAGGCTGCTGCA-3' (N represents an equimolar ratio of A, G, C and T) was annealed with a primer oligonucleotide 5'-GCAGCTGCGCTTCT-3'. Synthesis of the complementary strand was accomplished using DNA polymerase (Sequenase version 2.0; US Biochemical). A PstI sticky end was present after annealing and synthesis, and a HindIII sticky end was generated by restriction digestion. This semi-random cassette was then ligated into a newly constructed vector, pRW-2. This plasmid is a derivative of pEMBL-18 (ref. 35), into which the cytochrome b-562 gene³⁶, including its natural signal sequence, is expressed under *lac* control. The PstI site occurs naturally in the wild-type gene for cytochrome b-562. The HindIII site was introduced by changing the Leu₇₈ codon

(Wild type)
Glu-Gly-Lys Cys-Leu-Arg Leu-Ala-Ala Deletion

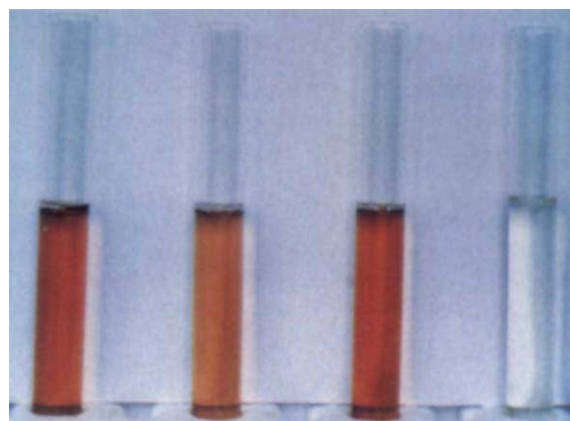


FIG. 2 Periplasmic fractions from *E. coli* cells expressing high levels of wild-type or mutant forms of cytochrome b-562. The red colour results from the accumulation of native folded haem-protein. The tube on the right represents a control experiment showing the periplasmic fraction from cells harbouring plasmid pRW-2, which contains a large deletion and a frameshift at the end of α -helix 3.

METHODS. Cultures of *E. coli* strain MV1190 harbouring the appropriate sequence derivative of the overexpressing plasmid were grown in 2 × YT medium to $A_{500}=1$. IPTG was added to a concentration of $100 \mu\text{g ml}^{-1}$ and growth was continued overnight. Cells were collected by centrifugation then washed in 50 mM Tris HCl, pH 8.0, 200 mM NaCl, and centrifuged again. The cell pellet was subjected to four cycles of freezing and thawing to disrupt the outer membrane, then taken up in water to release the periplasmic contents. Spheroplasts were removed by centrifugation, yielding a bright red supernatant with the characteristic absorption peaks of reduced cytochrome b-562 at 426 nm (the Soret band) and 562 nm. This solution was further enriched for cytochrome b-562 by adjusting the pH to 4.2 with sodium acetate (final concentration, 50 mM) and removing acid-insoluble contaminants by centrifugation. Extracts from cells harbouring the control plasmid pRW2 are colourless, indicating that background levels of cytochrome are negligible.

from CTG to CTT by site-directed mutagenesis³⁷. But in pRW-2, the wild-type HindIII-PstI fragment (encoding amino acids 77–91) has been replaced by a 20-bp 'dummy' fragment. In this fragment a frameshift is introduced, and a new restriction site (*Sal*I) is included. Use of the pRW-2 vector for constructing our library of turns has two advantages: (1) the true wild-type sequence does not enter the system except as one of the 8,000 possible sequences in the random library; and (2) after ligating the semi-random synthetic restriction fragment into the pRW-2 backbone, the ligation mix can be cut with *Sal*I to destroy any of the pRW-2 vector that may have re-closed without receiving a new synthetic piece.

Similar results were obtained for proteins with the turn sequences Pro₈₁-Val₈₂-Ala₈₃, Ile₈₁-Asp₈₂-Leu₈₃ and Tyr₈₁-Lys₈₂-Leu₈₃ (data not shown). In both oxidation states, the spectra of all four mutants are superimposable, within experimental error, upon the spectrum of the wild-type protein.

These results show that replacing the wild-type turn by a different sequence does not significantly perturb the structure of cytochrome b-562. Although minor changes in local structure

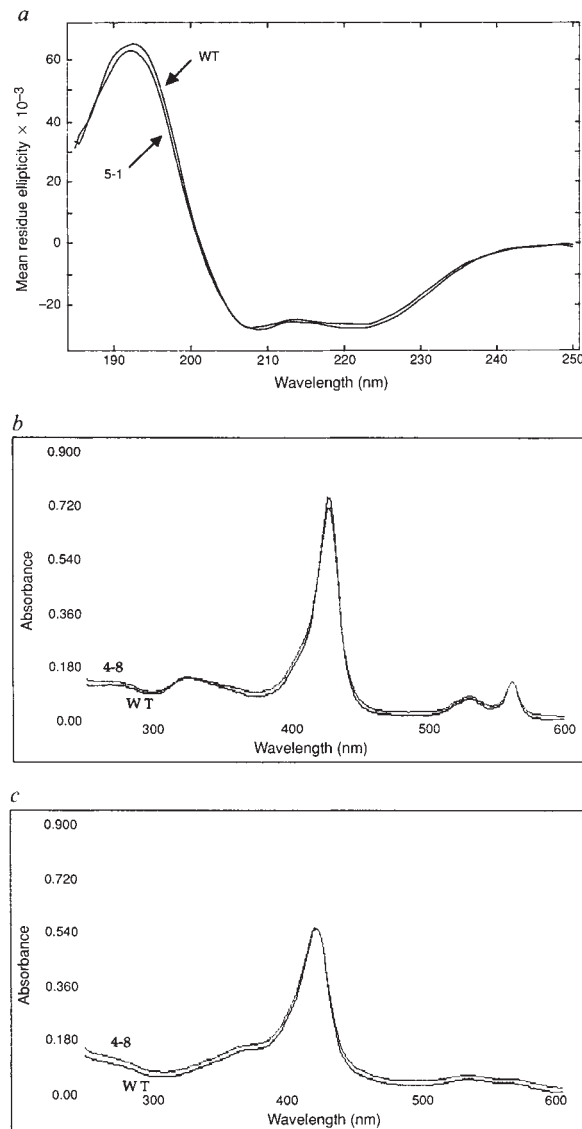


FIG.3 *a*, Circular dichroism spectra of wild-type and mutant cytochrome b-562. The wild-type turn sequence is Glu₈₁-Gly₈₂-Lys₈₃; the mutant (number 5-1) turn sequence is Pro₈₁-Val₈₂-Ala₈₃. Proteins were purified by ion-exchange chromatography over an S-Sepharose fast-flow column (Pharmacia) using a salt gradient from 0–1.0 M NaCl in a buffer containing 50 mM sodium acetate, pH 4.5. Before CD spectroscopy, proteins were dialysed into 10 mM sodium phosphate, pH 7.5, 50 mM NaF. Protein solutions were 5 μ M and optical path was 1 mm; spectra were recorded using an Aviv model 62DS instrument. The data were collected in triplicate from 260 nm to 185 nm with a step size of 0.5 nm. Similar results were obtained for the mutants Ile₈₁-Asp₈₂-Leu₈₃, Tyr₈₁-Lys₈₂-Leu₈₃ and Ser₈₁-Leu₈₂-Ser₈₃ (data not shown). *b* and *c*, Absorption spectra of reduced (*b*) and oxidized (*c*) forms of wild-type and mutant cytochrome b-562. The mutant 4-8 turn sequence is Ser₈₁-Leu₈₂-Ser₈₃. Conditions as in *a*, except that optical path length was 1 cm and spectra were recorded in a Hewlett-Packard 8452A diode array spectrophotometer. Dithionite (final concentration 0.5 μ g ml⁻¹) was used as reducing agent. Similar results were for mutant Ile₈₁-Asp₈₂-Leu₈₃, Tyr₈₁-Lys₈₂-Leu₈₃ and Pro₈₁-Val₈₂-Ala₈₃ (data not shown).

TABLE 1 Mutations in the third interhelical turn of cytochrome b-562

Red mutants

Mutant	DNA sequence*	Amino-acid sequence
Wild type	GAA.GGT.AAA	Glu - Gly - Lys
1-2	AGT.TCT.AGG	Ser - Ser - Arg
1-10	TTG.TGG.CGA	Leu - Trp - Arg
2-5	AAC.CTG.CGC	Asn - Leu - Arg
2-10	CGT.ACT.AAA	Arg - Thr - Lys
3-7	TTC.TTC.AGT	Phe - Phe - Ser
3-9	CTA.TTT.ATG	Leu - Phe - Met
3-10	AGA.GGA.ATG	Arg - Gly - Met
4-3	ATT.TCT.GCG	Ile - Ser - Ala
4-4	TAT.CTT.CAC	Tyr - Leu - His
4-5	GAC.TTT.CGT	Asp - Phe - Arg
4-6	TGC.CGG.GCG	Cys - Arg - Ala
4-7	TGC.TTC.ATG	Cys - Phe - Met
4-8	TCG.CTG.TCC	Ser - Leu - Ser
4-10	TTG.GGA.CGG	Leu - Gly - Arg
5-1	CCC.GTC.GCA	Pro - Val - Ala
5-3	TGT.TTG.AGA	Cys - Leu - Arg
5-5	AGG.GGA.GTT	Arg - Gly - Val
5-7	CTT.AAC.GCG	Leu - Asn - Ala
5-9	ATT.GAC.CTT	Ile - Asp - Leu
5-10	TAT.TAC.GAC	Tyr - Tyr - Asp
6-1	AGT.AGG.GAG	Ser - Arg - Glu
6-2	TTA.AGC.ATA	Leu - Ser - Ile
6-4	TCA.TGT.TTC	Ser - Cys - Phe
6-5	CTG.GCT.GCG	Leu - Ala - Ala
6-9	TAC.AAG.TTA	Tyr - Lys - Leu
6-10	TTA.TGG.CTA	Leu - Trp - Leu
7-5	TTT.GTT.AAC	Phe - Val - Asn
7-7	GCG.GTA.AAG	Ala - Val - Lys
7-9	AGC.ATG.CGC	Ser - Met - Arg
7-10	GAA.GAT.TTT	Glu - Asp - Phe
8-7	CAT.GAA.ACT	His - Glu - Thr

White mutants (stop codons)

1-3	TAG.TTA.TGT	STOP - Leu - Cys
1-5	TAG.AGC.GTG	STOP - Ser - Val
3-8	GTG.TAG.AGG	Val - STOP - Arg
5-2	AAG.TAG.TAT	Lys - STOP - Tyr
6-3	TGC.TAG.GTC	Cys - STOP - Val
6-7	CCG.TAA.TGG	Pro - STOP - Trp
7-1	CGT.TGA.GGA	Arg - STOP - Gly
9-3	GTA.TGA.TCT	Val - STOP - Ser
9-9	TTT.GTA.TAG	Phe - Val - STOP

Not listed are five deletion mutants that introduced frameshifts and were therefore discarded. Phagemid DNA was prepared by standard techniques³² using phage M13-K07 as helper phage. The sequencing primer is a synthetic oligonucleotide with the sequence 5'-AGTCCCTGTTACCTGAGTGC-3'. DNA was sequenced by the dideoxy method³³ using single-stranded phagemid DNA as template. We have analysed 32 randomly chosen sequences from among the 8,000 possible tripeptides. The probability (*P*) of observing the native-like fold in all 32 of them is

$$P = \frac{31}{\prod_{i=0}^{31} (R-i)/(8,000-i)}$$

where *R* is the total number of tolerated tripeptides in the collection of 8,000. In this formula, if *R* is less 7,286 (if less than 91% of the turn sequences give rise to native-like conformations), then the probability of randomly choosing 32/32 tolerated sequences would have been <5% (ref. 22). Thus we can say with 95% certainty that >91% of all possible tripeptides are tolerated.

around the turn are possible, the overall secondary structure of the 4-helix bundle and the specific tertiary structure around the haem must be similar in the wild-type and mutant proteins.

Our results indicate that the turn connecting helices 3 and 4 in cytochrome b-562 is not crucial in determining the folded structure of this protein. Although we cannot exclude the possibility that among 8,000 possible tripeptide sequences there exist some that prevent formation of the correct native structure, our results suggest that such sequences must be rare. A statistical analysis indicates with 95% certainty that >91% of all possible tripeptides are tolerated²² (see Table 1 legend).

It is important to consider the impact of these mutations on both the kinetics of protein folding and the thermodynamics of protein stability. Our experiments cannot rule out the possibility that some of the mutant proteins may fold at rates different from the wild type, but they do show that all 31 mutants fold sufficiently rapidly to escape proteolytic degradation *in vivo*; this indicates that for cytochrome b-562 there is no obligatory folding pathway requiring a specific tripeptide in the turn connecting helices 3 and 4.

For a turn whose side chains are exposed to solvent, sequence changes might be expected to influence stability either by introducing conformational strain in the folded state²³ or by affecting interactions with the solvent (in either the folded or unfolded state)^{24,25}. For example, replacement of the wild-type Gly 82 might strain the native structure, because the ϕ and ψ angles of this glycine (79° and 10°) fall in a region of the Ramachandran plot²⁶ rarely observed for other residues²⁷. However, substitution of this glycine by virtually any other amino acid can be tolerated (Table 1), indicating that the region around residue 82 can relax to accommodate turn conformations different from the wild-type.

Replacement of Glu 81 or Lys 83 might be expected to destabilize the protein by disrupting a favourable electrostatic interaction between these residues. But it is unlikely that such an interaction contributes to stability because these side chains point in opposite directions in the crystal structure. Neither does the interaction of these charged residues with the solvent contribute to the stability of the protein, because many different side chains can be tolerated at positions 81 and 83 (Table 1). Even in the extreme case in which the substituents are Ile 81 and Leu 83 the protein is stable and its T_m only slightly reduced (A.P.B., E.S.H. and M.H.H., manuscript in preparation).

We conclude that individual interhelical turns play only a minor part in determining protein structure. Although our mutant tripeptides undoubtedly differ in their intrinsic tendencies to form particular structures in solution, they can all be constrained to adopt conformations consistent with a properly folded structure. This finding argues that local effects can be overcome by long-range interactions between residues not adjacent in sequence. The tolerance of interhelical turns to alterations in sequence, even of different hydrophobicity patterns, has implications not only for our understanding of natural proteins, but also for the design of novel proteins²⁸⁻³¹ and for designing active sites into engineered proteins. □

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Group II self-splicing introns in bacteria

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LIKE nuclear premessenger introns, group II self-splicing introns are excised from primary transcripts as branched molecules, containing a 2'–5' phosphodiester bond. For this reason, it is widely believed that the ribozyme (catalytic RNA) core of group II introns, or some evolutionarily related molecule, gave rise to the RNA components of the spliceosomal splicing machinery of the eukaryotic nucleus¹. One difficulty with this hypothesis has been the restricted distribution of group II introns. Unlike group I self-splicing introns, which interrupt not only organelle primary transcripts, but also some bacterial and nuclear genes^{2–5}, group II introns seemed to be confined to mitochondrial and chloroplast genomes (reviewed in ref. 6). We now report the discovery of group II introns both in cyanobacteria (the ancestors of chloroplasts⁷) and the γ subdivision of purple bacteria, or proteobacteria⁸, whose α subdivision probably gave rise to mitochondria⁹. At least one of these introns actually self-splices *in vitro*.

As a first step in our search for bacterial group II introns, we attempted to design primers that would allow polymerase chain reaction (PCR) amplification of group II coding sequences. Unfortunately, of the six structural domains that constitute the ribozyme core of group II introns⁶, only domain V is sufficiently conserved in sequence to allow design of a degenerate oligonucleotide (RID-1; Fig. 1) that is likely to hybridize to most of the known members of group II. To obtain a second primer (RID-2), we exploited the facts that some group II introns contain long open reading frames (ORFs) and the proteins translated from these ORFs tend to share seven blocks of conserved

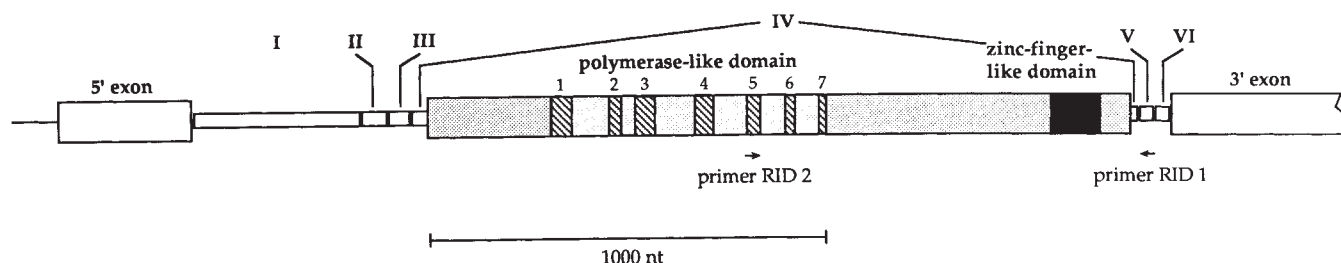


FIG. 1 Organization and PCR amplification of protein-encoding group II introns. The figure was drawn from the sequence of the Cal.x1 intron of *Calothrix* (Fig. 2), whose organization is typical of other protein-coding members of group II. The split exonic ORF is shown as large empty boxes, the intron-contained ORF as a large grey box, with the putative polymerase domain^{10,11} and a zinc-finger-related domain indicated (numbers refer to the 7-amino-acid motifs defined in ref. 10). Small boxes labelled I to VI symbolize ribozyme structural domains⁶. RID-1 (complementary to the 3' half of ribozyme domain V) and RID-2 (derived from the consensus coding sequence of polymerase motif 5) are the primers used for PCR amplification.

METHODS. The following bacterial strains were tested for PCR amplification. Proteobacteria: *E. coli* isolates 19 and 61 from the ECOR collection¹⁷, *Agrobacterium tumefaciens* B6 and C58, *Rhizobium meliloti* Mr41 and *Azotobacter vinelandii* UWR. Cyanobacteria: *Calothrix*

PCC7601 and PCC7101 and *Synechocystis* strains PCC6714 and PCC6803. Bacterial cultures: purple bacteria were grown in LB medium¹⁸, cyanobacteria were grown in Herdman medium¹⁹ with twice the standard nitrate concentration. DNA was extracted by the hexadecyltrimethyl-ammonium bromide procedure¹⁸ and treated with RNase A. PCR amplification was carried out with Taq polymerase using 0.2 mM of degenerate primers RID-1 (5'-TCCCTCCGAACCGTACGTG(C/A)NA(G/C)T(T/C)TC) and RID-2 (5'-ACCGTATACGT(A/C)GNTA(C/T)GCNGA(T/C)GA) and 25–100 ng bacterial DNA in 10 mM Tris-HCl, pH 8.8 at 25 °C, 1.5 mM MgCl₂, 50 mM KCl. The annealing temperature was 35 °C for the first 10 cycles and then raised to 45 °C. Amplification products were gel-purified and cloned into plasmid pTZ19U for double-strand sequencing by the dideoxy chain termination method (Sequenase, US Biochem.).

sequence with the polymerase domain of reverse transcriptases^{10,11} (Fig. 1).

As the probable progenitors of mitochondria and chloroplasts, proteobacteria and cyanobacteria were reasonable candidates for possession of group II introns. Of six proteobacterial strains tested (Fig. 1), only *Azotobacter vinelandii*, which belongs to the γ subdivision¹², gave rise by PCR amplification of DNA extracts to an abundant amplification product. Multiple major bands, as well as additional minor products, were also obtained from the cyanobacteria *Calothrix* PCC7601 and PCC7101. As would be predicted should the amplified DNA be derived from a *bona fide* group II intron, subsequent cloning and sequencing revealed the presence of the consensus sequence for the 5' half of ribozyme domain V at the RID-1 end of the major PCR products from *A. vinelandii* UWR and *Calothrix* PCC7601 (two distinct amplification products were recovered from the latter extract). By contrast, the minor products lacked both this motif and any recognizable polymerase domain at their RID-2 end.

One of the two *Calothrix* PCC7601 putative introns (designated Cal.x1), as well as sections of the *A. vinelandii* putative intron (AvUWR.x1), were sequenced from clones isolated by probing DNA libraries of the host genomes (Fig. 2). The Cal.x1 sequence includes a 1,752-nucleotide ORF with extensive similarity to the ORFs contained in organelle group II introns. Consistent with the conserved organization of protein-encoding group II introns (Fig. 1), sequences upstream and downstream of that ORF can be folded into the characteristic structures of domains I to III and V and VI, respectively, of a group II ribozyme core (Fig. 2a). As also expected, domain IV is generated by the pairing of complementary sequences at the two ends of the ORF. Partial sequencing of the second putative intron in *Calothrix* (Cal.x2) showed both its potential RNA structure over domains V and VI and protein-coding sections to be closely related to those of Cal.x1 (Fig. 2). Available sequences from the *A. vinelandii* clone are more divergent and appear to lack motifs 6 and 7 of the polymerase domain, but the remaining sections can easily be aligned with the sequences from *Calothrix* (Fig. 2c).

Ability of the putative ribozyme core in the Cal.x1 sequence to direct self-splicing *in vitro* was investigated (Fig. 3). A two-

hour incubation resulted in conversion of some precursor molecules into products with the expected lengths for linear intron, 5' exon, 3' exon and ligated exons (Fig. 3a). The identity of the latter product was verified by sequencing: the observed intron-exon junctions (Fig. 3b) coincide precisely with those independently inferred from the ribozyme core model (Fig. 2; note also that splicing results in putting the 5' and 3' exon ORFs into phase). Also evident among the splicing products was a molecule whose reduced mobility (Fig. 3a) and inability to be reverse-transcribed beyond the 5' splice junction (Fig. 3c) makes it a likely candidate for the expected branched circle (lariat).

Our results indicate that group II introns are probably widespread, at least in the bacterial groups tested, and particularly as our search strategy was by no means exhaustive: (1) non-protein-coding introns were excluded; (2) it is unknown whether organelle group II introns, from which the sequences of our degenerate primers were derived, are representative of the true diversity of group II members; (3) competition for PCR amplification among different introns from the same host could lead to gross overrepresentation in the final product of those sequences with best match to the consensus.

A question raised by our results concerns the origins of bacterial group II introns. Like many group I introns¹³, some protein-encoding mitochondrial group II introns are mobile, in the sense that they practise 'homing', that is, the invasion of intronless copies of the genes in which they are inserted^{14,15}. Although this makes group II introns prime candidates for horizontal transmission, there is nothing to suggest a recent organelle origin for the *Calothrix* and *Azotobacter* introns. First, the Cal.x1 and *A. vinelandii* introns have base compositions (41.5% and 57% (G + C), respectively) that are rather similar to those of *Calothrix* and *A. vinelandii* genes in the data banks, just as should be the case if these introns were indeed long-term residents of their bacterial hosts. Then, the very similar structure of ribozyme domain VI in the three bacterial introns is distinctive enough to set them apart from all other known members of group II (ref. 6, and our unpublished data). Finally, no conclusion can be drawn from the fact that similar zinc-finger-like sequences exist at the 3' end of the three bacterial intronic ORFs and the 3' end of the ORF in the *S. obliquus* algal group II intron¹⁶, because a

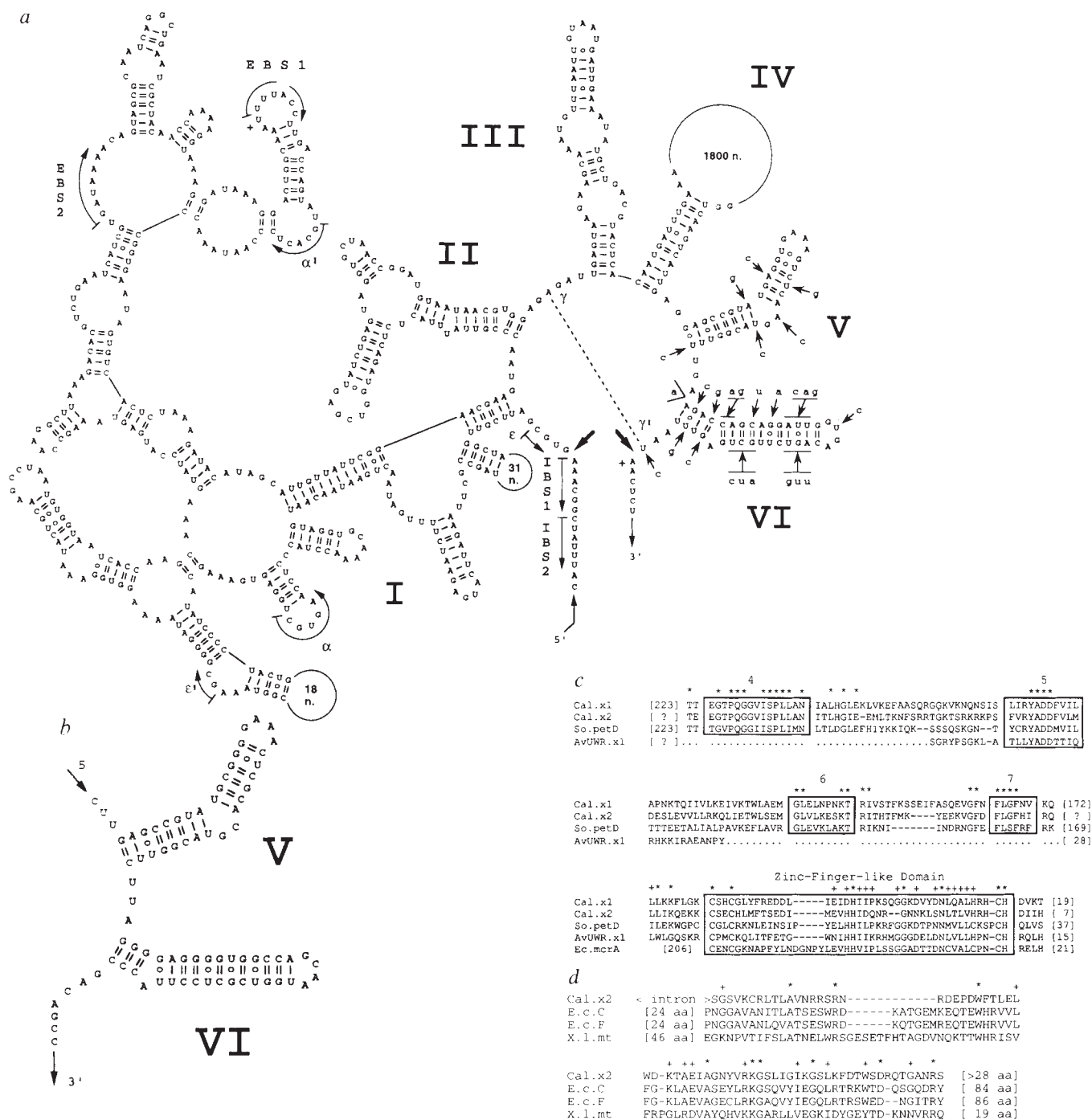


FIG. 2 Intron sequences and secondary structures. **a**, Structure of the ribozyme core of the Cal.x1 intron, established by comparative analysis⁶. Tertiary pairings common to most group II introns^{6,20} are indicated by arrows (α - α' , ϵ - ϵ' , EBS1-IBS1, EBS2-IBS2), a dashed line (γ - γ') or (+) signs. Heavy arrows point to splice junctions, as inferred from secondary and tertiary pairings. Changes in the Cal.x2 sequence are in lower case (domains V and VI only). **b**, domains V and VI from the AvUWR.x1 sequence. **c**, alignment of sections of the bacterial intron-contained ORFs with corresponding sections of the *Scenedesmus obliquus* (alias KS3/2) *petD* intron¹⁶ and the 3' terminal section of the *mcrA* protein of *E. coli*²¹. Numbered boxes correspond to conserved motifs in the polymerase domain¹⁰, numbers in square brackets to amino-acids not shown, asterisks and crosses to sites of complete or partial (for the zinc-finger-related domain only) sequence conservation. **d**, Alignment of the sequence translated from the ORF 3' of the Cal.x2 intron with single-stranded DNA-binding proteins from *Xenopus laevis* mitochondria (X.1. mt)²² and the chromosome E.c. C and F-plasmid (E.c. F) of *E. coli*²³. Asterisks designate amino acids common to all proteins, and crosses, those common to the three bacterial proteins. The prob-

ability of the Cal.x2 versus *E. coli* C similarity being due to chance was estimated to be 6.7×10^{-5} by the BLAST program²⁴ at the National Center for Biotechnology Information (NIH, USA).

METHODS. DNA libraries of *Calothrix* PCC7601 and *A. vinelandii* UWR obtained by standard procedures¹⁸ were screened with ³²P-labelled oligonucleotides specific for the Cal.x1 and AvUWR.x1 amplification products. The sequence (Fig. 1) of a 3,330-bp section of the pCal11-positive clone was determined on both strands by standard procedures¹⁸ (accession number X71404). Partial sequences were generated from an *A. vinelandii* positive clone, using primers annealing to the DNA amplification product. Sequences of the exon 3' to the Cal.x2 intron, its polymerase motif 4 and ribozyme domains V and VI, were obtained by *Eco*RI digestion of *Calothrix* PCC7601 DNA, ligation at low DNA concentration and divergent PCR amplification using primers annealing to the ends of the RID-1/RID-2 amplification product (Fig. 1).

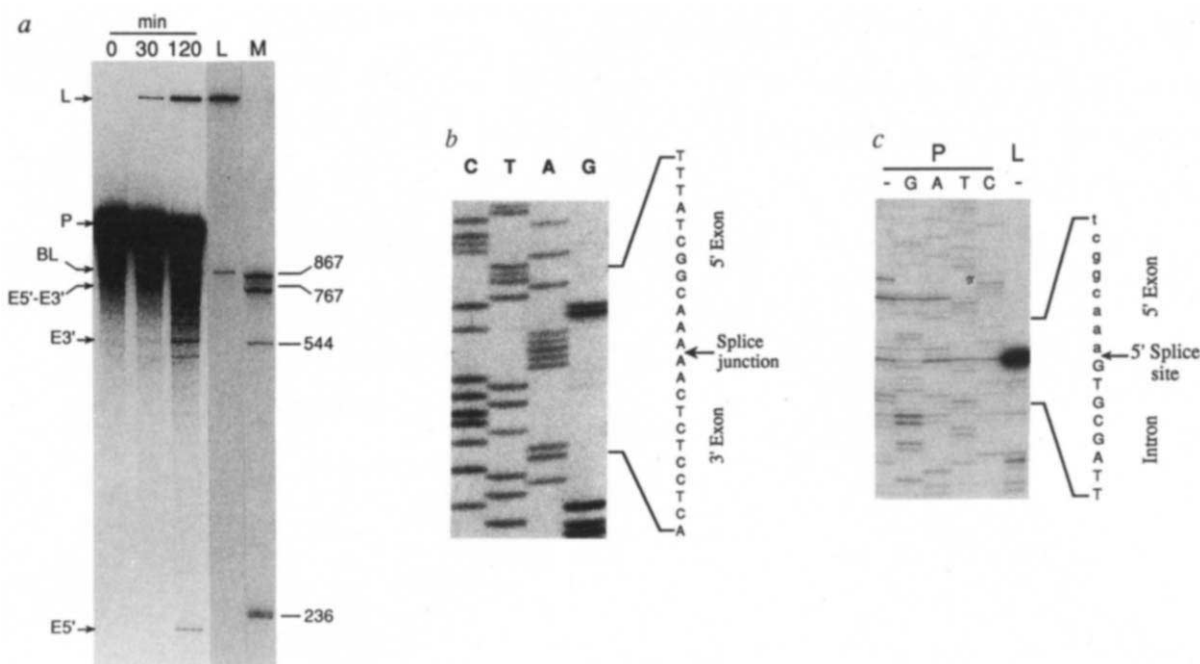


FIG. 3 *a*, Self-splicing reaction of intron Cal.x1. After incubation of precursor RNA for specified times, samples were loaded on a 4% polyacrylamide-8 M urea gel. Bands with the expected migration behaviour for precursor (P, 1,650 nt), lariat intron (L), broken lariat intron (BL, 875 nt), 5' exon (E5', 231 nt), 3' exon (E3', 544 nt) and ligated exons (E5'-E3', 775 nt) are labelled accordingly. Lane L, migration on the same gel of putative lariat molecules that had been incubated for 45 min at 30 °C after purification. Because the 2'-OH group that initiates group II self-splicing is located only 7-8 nucleotides upstream of the 3' splice junction⁶, branched molecules resulting from random opening of the circular section of the excised intron lariat should migrate to about the same position as the linear intron. Lane M, RNA size markers. *b*, Sequence of ligated exons. Lanes are labelled with the complement of the dideoxynucleoside triphosphate. *c*, Primer extension on gel-purified, putative intron lariat (lane L). The sequence on the left was generated by reverse transcription of gel-purified precursor RNA (P). Lanes are

labelled as in *b*. A minus sign indicates no dideoxynucleoside triphosphate was added.

METHODS. Plasmid pCalx1Δ1575 derives from plasmid pCal11 by deletion of ORF sequences from intron positions 660 to 2,234 and removal of all but the last 220 nucleotides of the 5' exon. Precursor RNA was generated by transcription of *Eco*RI-digested pCalx1Δ1575 DNA with T7 RNA polymerase followed by gel purification²⁵. Self-splicing conditions: RNA (4 nM) was incubated at 45 °C in 40 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 500 mM NH₄Cl²⁶. Sequencing of ligated exons was gel-purified and reverse-transcribed¹⁸ with primer 5'-GACCAAGGTA-GTGGCACTTCACA-3'. The cDNA was PCR-amplified with primers 5'-AAAGATTTCATGTAGTTCGATGTT and 5'-CAAACCTATGAACCTATCCAATA specific for the 5' and 3' exons, cloned and sequenced. The same conditions were used for reverse transcription of gel-purified precursor RNA and putative intron lariat (75 fmol), with primer 5'-GTTATCAAAGA-TTTCAT, hybridizing to intron positions 65-83.

section of the *mcrA* protein from *Escherichia coli* is just as closely related (Fig. 2c). It could therefore be that group II introns are not only widespread in bacteria, but were present in their common ancestor.

A probable benefit from the lasting presence of introns is regulated slicing. Whereas the ORF 3' of the Cal.x2 intron encodes a product related to single-stranded-DNA-binding proteins (Fig. 2d), we have been unable to find cognates of the ORFs flanking the other *Calothrix* intron or the *Azotobacter* intron in data banks, and have no clues as to their possible functions. Fortunately, those questions, as well as other matters pertaining to the biological roles(s) of group II introns, are now amenable to the powerful methods of bacterial genetics. □

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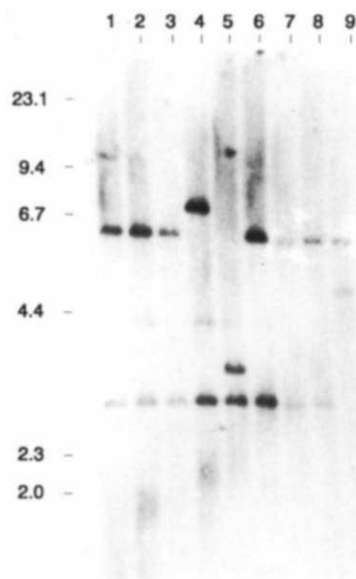
ACKNOWLEDGEMENTS. We thank C. Astier and C. Vernotte for cyanobacterial cultures, H. Ochman for his *ECOR* collection of reference strains, and Y. Dessaux for other proteobacterial strains.

The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases

D. Vetriche et al.

Nature **361**, 226–233 (1993).

In Fig. 2b of this article, the sizing of DNA fragments was incorrect. The corrected sizes are shown below, together with the appropriately amended section of the legend to Fig. 2c.



Section D (which represents the 5' end of the *atk* cDNA sequence) detected 0.7, 1.7, 1.8, 2.5 and 6.2 kb *Msp*I fragments in normal genomic DNA; in patient E, no 6.2 or 2.5 kb *Msp*I fragments were observed; a novel 7.2 kb band was seen. cDNA 6G11 (which does not extend to the 5' terminus of the *atk* gene) hybridized to 0.7, 1.0, 3.0, 4.3 and 6.2 kb *Msp*I fragments in normal individuals and also detected the abnormality in patient E. Section C detected no *Msp*I abnormalities in patient E. Section C detected 1.0, 1.7, 3.0 and 4.3 kb *Msp*I fragments in normal DNA; the 3.0 kb fragment was absent in patient D, but a novel 1.2 kb band was observed. Section D did not detect any abnormalities in patient D.

Atomic structure and chemistry of human serum albumin

X. M. He & D. C. Carter

Nature **358**, 209–215 (1992)

WE failed to recognize the following typographical error in the proofs of this research article. In Table 1, page 211, the unit cell constant for the *b* axis of the recombinant crystal form should have been 88.3 Å instead of 38.3 Å. A statement in Fig. 4 should

read that Lys 199 and His 242 are located at the 'top' of Fig. 4a, not the 'bottom'. In addition, we incorrectly referenced an earlier publication on recombinant albumin (ref. 25). The appropriate reference should be: Sleep, D., Belfield, G. P. & Goodey, A. R. *Biotechnology* **8**, 42–46 (1990). □

Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis

Daniel R. Rosen et al.

Nature **362**, 59–62 (1993)

FIGURE 1c in this letter shows a comparison of amino-acid sequences for exon 2 of SOD-1, but incorrectly positions the residue numbers and the mutations on the sequences. The corrected panel is shown here.

EXON 2

[illegible]

Also, in Table 3, the base-pair change for family 9967C should read GGC→CGC. $\square$

A single amino acid of the cholecystikinin-B/gastrin receptor determines specificity for non-peptide antagonists

**Martin Beinborn^{*†}, Young-Mee Lee^{*},
Edward W. McBride^{*}, Suzanne M. Quinn^{*}
& Alan S. Kopin^{*‡}**

Nature **362**, 348–350 (1993)

WE have noted an error in our numbering of amino acids in this letter. The valine in transmembrane domain VI that confers antagonist affinity to the human receptor was erroneously numbered as 319. The correct numbering should be human valine 349. In addition, the numbering of three of the canine mutants shown in Fig. 2 is incorrect: ^{316}G should be ^{322}G , ^{321}Y should be ^{327}Y , and ^{360}SS should be ^{366}SS . The relative positions of the human and canine amino-acid sequences shown in Figs 2 and 4 are correct; the numbering error therefore does not affect any of our conclusions. $\square$

Breaking up is not hard to do

Among this week's tools for separation — a modular capillary electrophoresis system with in-built autosampler, chromatography data systems, and columns for chiral separations and the separation of T and B lymphocytes.

The Camag **thin-layer chromatography cabinet** is designed to remove excess spray mist when reagents are being sprayed on a thin-layer chromatogram (*Reader Service No. 100*). Use of the cabinet ensures the



Camag's TLC spray cabinet removes excess aerosol.

complete removal of excess spray from atomizer and spray particles rebounding from the TLC plate. The spray cabinet is also useful for removing solvent vapours from a freshly developed plate. The bottom of the spray cabinet has a built-in tray that can be removed for easy cleaning.

Hitachi Scientific has news of a new application for its F-4500 rapid-scan spectrophotometer — the measurement and characterization of HPLC eluants (*Reader Service No. 101*). The company says that the use of a **scanning fluorescence spectrophotometer** retains the increased sensitivity of fluorescence methods compared to ultraviolet detectors, yet provides more information than can be obtained from conventional fixed-wavelength fluorescence detectors. The availability of the complete spectrum of the eluting compound allows it to be characterized to confirm that it is the expected compound. In addition, spectra of the eluant can be recorded at different times during the chromatogram as the rapid wavelength scan speed of the F-4500 (30,000 nm min⁻¹) will generate a spectrum over a 250-nm wavelength range in about 0.5 s. Hitachi says that this allows the purity of the eluting peak to be monitored throughout the separation. The data sampling interval of the F-4500 can be as small as 1 ms. This fast sampling rate gives an effective signal inte-

gration during the rapid scan. The instrument is controlled using the Windows graphical interface.

■ Electrophoresis

The Crystal 310, part of the Crystal 300 Series of modular **capillary electrophoresis systems** from ATI Unicam, incorporates a 48-position autosampler carousel within the modular injection system, making the system suitable for both routine analyses and methods development (*Reader Service No. 102*). Both large 4-ml vials and 300- μ l micro-vials are available. A vial-capping system and Peltier cooling (4–40 °C) of the carousel are used to prevent evaporative loss during multiple analyses. The Crystal 310 incorporates the novel Dynamic Compression Injection and pressure and voltage ramping systems that provide a reproducibility of injection of less than one per cent, according to ATI Unicam. Both electro-



ATI Unicam's capillary electrophoresis system has a modular make-up.

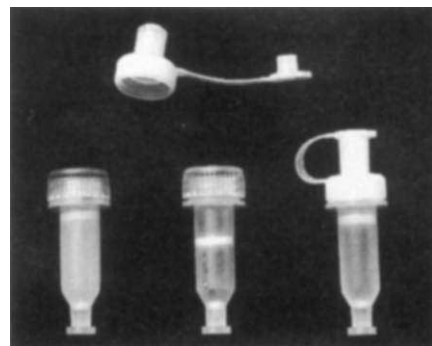
kinetic and pressure injection modes are offered. Because of the system's modular design any detector can be used. However, the company offers a choice of ultraviolet/visible, diode-array and fluorescence detectors. The Windows-based multitasking data system offers five standard analysis options, offering flexible analysis routines for both online and re-analysis.

SigmaMarker **protein standards**, which are specially designed for use as standards in the PhastSystem electrophoresis workstation and standard SDS-PAGE laemmli systems, are now available within high-, low- and wide-molecular-weight ranges from Sigma (*Reader Service No. 103*). The high-molecular-weight range SigmaMarker contains eight proteins ranging from 36,000–205,000 daltons in size; the low-molecular-weight range contains proteins from 6,500–66,000 daltons in size. The wide-molecular-weight range SigmaMarker contains 13 proteins from 6,500–205,000 daltons in size. Sigma says that these products are provided in a stable, lyophilized form

and only require reconstitution with deionized water before loading on a gel.

■ Filtration

Push columns from Integrated Separation Systems are **miniature gel filtration columns** that are suitable for the purification or desalting of small, precious samples (*Reader Service No. 104*). Available pre-



Gel filtration columns in miniature.

packed with either GF-25 resin (for oligos and proteins) or GF-50 resin (for nick translations), each autoclaved column contains 0.8 ml of gel bed equilibrated in DNase- and RNase-free water. The top and bottom of the bed are fitted with porous polyethylene frits to protect the gel during storage and use. The columns can be operated with a 1-ml syringe. Each column is supplied with a tip cap, storage cap and luer cap. Empty columns include top and bottom frits.

Schleicher & Schuell's Centrex **small-volume microfilters** are now available in a 40- μ l volume for the elution of DNA from agarose gels (*Reader Service No. 105*). The Centrex MF-4 400 μ l microcentrifuge filters consist of a polypropylene filter insert and a 1.9-ml receiver tube and cap that fits into a standard microcentrifuge. Available



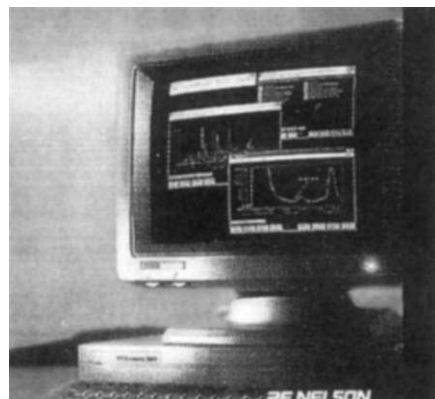
Small-volume microfilters from S & S.

with a low-protein-binding cellulose acetate membrane, the Centrex MF-4 is designed to replace harsh extraction methods and minimize sample handling. In addition, the filters are available with chemically resistant nylon membranes for small volume (that is 20–400 µl) HPLC sample preps.

■ Chromatography data handling

Gynkotek HPLC has recently added two software modules to the GynkoSoft chromatography data system range for use with the company's high-sensitivity diode-array detectors (*Reader Service No. 106*). The first adds on to base packages and is aimed at providing automated peak purity analysis to the routine or high-sample-throughput laboratory. The other module provides peak confirmation and identification via a user-defined spectrum library. Gynkotek says that both packages are designed for use either interactively, enhanced by graphics, or in fully automated batch mode.

Perkin-Elmer Nelson's ACCESSCHROM multitasking, multiuser chromatography system may be just the job for analytical laboratories that need a centralized approach to chromatography data processing (*Reader Service No. 107*). Running on the



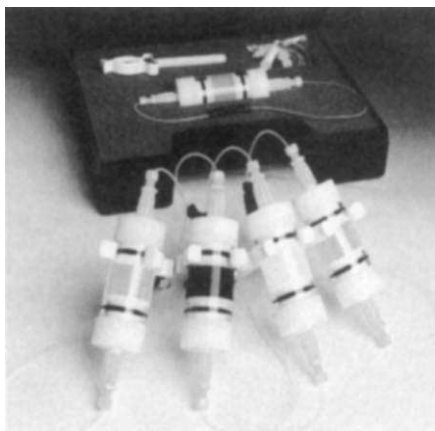
VAX-based data handling system.

DEC VAX family of computers, the system covers everything from chromatography method and sequence creation, data channel collection, reintegration and post-run data manipulation through graphics, and chromatogram comparison, ratio and difference. A 12-page brochure that highlights the new system describes how each VAX system can support the individual activities of many users simultaneously, providing consistency for the comparison of data, remote access using Ethernet and a level of control that allows users to create and edit software methods. Multitasking capabilities include real-time data acquisition, simultaneous multiple operation and communication with other VAX systems. Chromatography data from all commercially available chromatographs can be collected on the ACCESSCHROM system through either the PE Nelson Model 941 intelligent interface or the

Series 600 LINK interface. The system's custom report generator allows users to tailor reports to suit their own needs.

■ Column collection

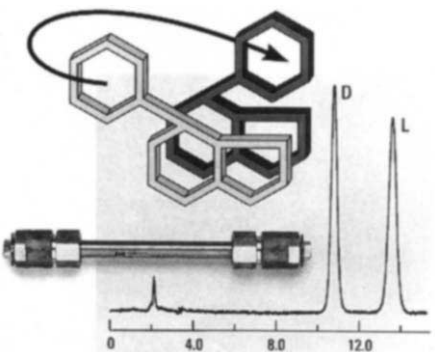
Affinity Chromatography has now made its range of Mimetic and hydrophobic interaction chromatography (HIC) adsorbents for protein separations available as prepacked



Alpha columns for affinity and hydrophobic interaction chromatography.

Alpha columns (*Reader Service No. 108*). With a fixed 10-ml bed volume and operational flow rates to 20 ml min⁻¹, the company says that the Alpha columns offer linear scale-up direct from its PIKSI and PIKSI-H adsorbent screening kits, and are compatible with most biochromatography systems. All Mimetic and HIC adsorbents are available in larger individual packs for laboratory-scale separations and in bulk for process applications.

For routine chiral separations, Hewlett-Packard recommends ChiraDex — its new chiral cartridge that is packed with a versatile silica-gel packing (*Reader Service No. 109*). The company says that enantiomeric



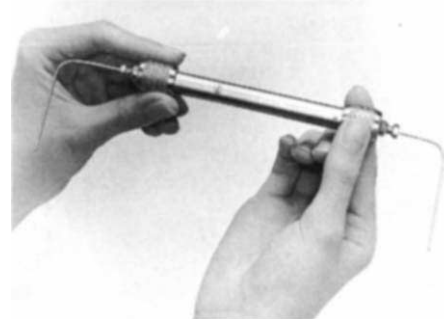
ChiraDex cartridges for chiral separations.

separations are possible using simple, non-chiral solvents, including simple mobile phase solvents. Separations can be optimized by adjusting buffer concentrations, pH and temperature. ChiraDex cartridges are suitable for a broad range of applications due to their β-cyclodextrin stereochemistry. Common compounds that have been sepa-

rated successfully with ChiraDex include barbiturates, benzothiadiazines, benzodiazepines, calcium antagonists, clor-thalidone, dansylamino acids, hydantoins, tribendilol, tropic acid and others.

Polyspher IC AN-1 is the new HPLC column from E. Merck that is designed for anion separations (*Reader Service No. 110*). As the ion exchange resin, a hydrophilic polymethacrylate with bonded quaternary ammonium groups, has a wide range of usable pHs it can be used to separate most inorganic anions and many organic anions (organic acids such as acetate, citrate, lactate or malate). The company says that standard anions such as bromide, chloride, fluoride, iodide, nitrate, nitrite, phosphate, sulphate, sulphite, thiosulphate or thiocyanate can be resolved, as well as special anions such as arsenate, bromate, chlorite, chromate, cyanide, molybdate, silicate and tungstate or polythionates or hypophosphite, phosphite and polyphosphates. By using a newly developed acidic eluent, E. Merck says that it is possible to separate the standard seven anions — bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulphate — without the interference of carbonate. Detection of anions is in the parts-per-billion range. Polyspher IC AN-1 is designed for single column anion chromatography and, therefore, can be connected to most HPLC systems. Anions are detected by conductivity or indirect ultraviolet measurement.

Chrompack's Chrompack 8 haemoglobin column is a HPLC column that is designed to separate HbA1c from the other



Haemoglobin analysis column.

haemoglobins present in haemolysed blood (*Reader Service No. 111*). The normal concentration of HbA1c lies around 6 per cent; a higher than normal amount of HbA1c indicates that the patient has had periods of hyperglycaemia (high blood sugar levels) in the previous 2–3 months. Where direct glucose measurement can provide only an outline sketch, the company says that its Chrompack 8 haemoglobin column provides a complete blood sugar profile over an extended period. A throughput of six samples an hour can be achieved by this method.

Silica-based packings have proven to be useful for a wide variety of HPLC analyses.

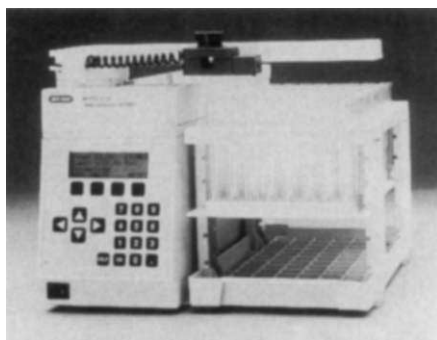
However, acidic, basic and zwitterionic compounds can interact differently with residual silanols to cause such problems as peak symmetry, retention time drift and irreproducible separations. Using special bonding and surface deactivation technology, Supelco has developed new Supelcosil LC-ABZ columns for analysing acids, bases and zwitterions (*Reader Service No. 112*). Through electrostatic shielding, an embedded polar group in the reversed phase silica-based packing of this column prevents silanol interaction with both acids and bases. Supelco says that, as a result, the column can be used to analyse polar, neutral, nonpolar, acidic or basic compounds — or mixtures of such components. Supelcosil LC-ABZ columns are said to provide good peak shapes at neutral pH and low ionic strength — without amine modifiers or other ion suppression techniques.

Eldan Tech's Uni-Sorb columns, available through Semat, are designed to provide a simple and quick way of separating T-enriched and B-enriched cell fractions from mixed lymphocyte populations (*Reader Service No. 113*). Specially treated nylon wool to which the B cells adhere, but the T cells do not, is packed into each sterile, ready-to-use Uni-Sorb tube. The top of the column is closed with a silicon rubber plug, and cell suspension is injected into the column through this. After incubation, the column is held upright and outflow is controlled by a special twist valve. According to the manufacturer, yields of more than 90 per cent can be expected when using the columns.

The BPX70 stationary phase from SGE is intended for the analysis of carbohydrates (*Reader Service No. 114*). It can be used in the separation of alditol acetates and separates complex mixtures of partially methylated alditol acetates derived from saccharide residues. BPX70 is a silphenylene-modified cyanopropyl polysiloxane stationary phase with equivalent polarity to 70 per cent cyanopropyl. SGE says that it is one of the few high cyanopropyl stationary phases compatible with the widely used hybrid spectroscopic technique of gas chromatography/mass spectrometry. Being chemically bonded and thermally modified with the silphenylene unit, the company says that column bleed of the BPX70 is held to a minimum, even at elevated temperatures more commonly achieved on nonpolar columns.

Fraction collectors

Designed for use with high- or low-pressure chromatography systems, analytical or preparative applications, the new Model 2128 fraction collector from Bio-Rad utilizes an x-y motion, two interchangeable racks and two adapters, and collects volumes from microlitres to litres at flow rates up to 100 ml min⁻¹ (*Reader Service No. 115*). The



Bio-Rad's multi-format fraction collector.

Model 2128 allows collection into standard test tubes using the standard rack or, by using adapters, it is possible to collect fractions into microtitre plates, capless microtubes or bottles of any size. The menu-driven software allows users to program the fraction collector with a variety of different collection methods, including an entire run, within time windows, by ultraviolet threshold or a combination of time windows and threshold.

Isco's new Foxy Jr fraction collector sizes fractions by time, drop or pumped volume; locates liquid chromatography and HPLC peaks; and collects fractions in the most useful way for most types of chromatography (*Reader Service No. 116*). By monitoring the slope or level of an ultraviolet or other detector signal and combining peak detection with selectable time windows, Foxy



Isco's Foxy Jr sizes fractions by time, drop or pumped volume.

Jr collects only desired peaks and diverts other effluent to waste to minimize the handling of glassware. Foxy Jr's space-saving design provides an overall footprint of only 27 × 31 cm but a capacity of up to 144 tubes. Interchangeable racks hold test tubes from 12 to 25 mm in diameter and up to 150 mm tall; full-size or mini-size scintillation vials; or microcentrifuge tubes. The fraction collector is suitable for use in a cold room.

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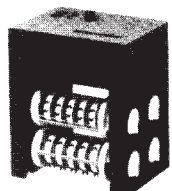
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■ Pumps and detectors

The LC Star 9002 and 9012 **HPLC pumps** from Varian feature a higher flow rate (of 10 ml min⁻¹ max.) for semi-preparative applications (*Reader Service No. 117*). Users can choose from three proportioning modes; this allows pump performance to be tailored



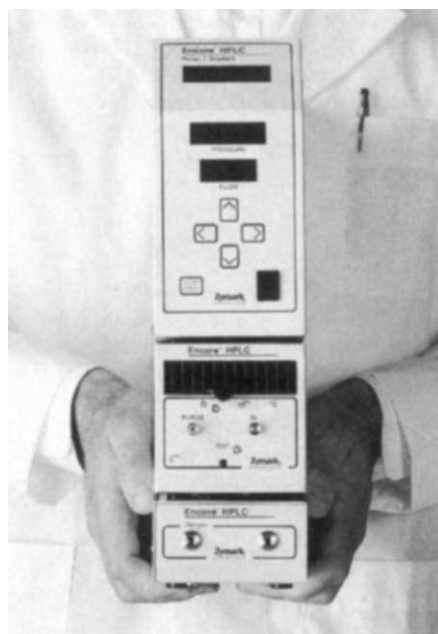
Varian's LC Star series of HPLC pumps.

to meet specific needs. The Star 9012 and Inert 9012 ternary gradient pumps feature a single piston design, with in-line, high-speed proportioning valves that are designed to produce repeatable mobile phase regardless of flow or composition. A mechanical inlet valve, rapid refill, and high-pressure mixing are design features that eliminate the need to sparge or de-gas solvents. Completing the line-up is the 9002 isocratic pump, which is based on the 9012 design.

Detection at two standard wavelengths — 254 nm and 280 nm — is a feature of the 112 **fixed-wavelength, ultraviolet/visible detector** from Gilson Medical Electronic (*Reader Service No. 118*). Additional premounted, prefocused lamp/filter assemblies are available that allow detection at

214 nm, 229 nm and in the range of 340–640 nm. With a 10-mm path length analytical flow cell, the stated sensitivity range of the 112 detector is 0.001–1.0 AUFS. Noise is less than 4×10^{-5} AU peak-to-peak (at 254 nm) for accuracy in trace analyses. There are 10 different sensitivity settings to choose from. A choice of six different flow cells allows users to match a flow cell to their sample load to prevent detector overload and off-scale peaks.

Zymark's Encore **HPLC pump** features a 10-cm wide footprint, a single-head design, innovative plunger/seal and valve designs and diagnostic software that ensures



Encore HPLC pump — new from Zymark.

precise flow, self-priming, and detection and correction for bubbles (*Reader Service No. 119*). The company says that for gradient use, the Encore HPLC delivers the right blend with every piston stroke using dynamic blending and without any mixers.

■ In a spin

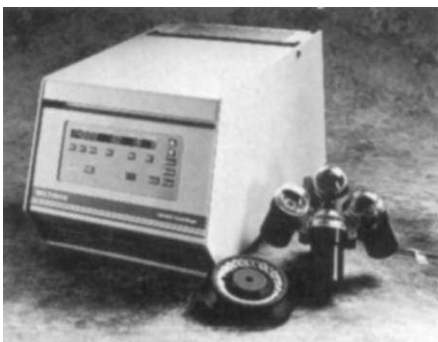
The new GS-15 series multipurpose **micro-centrifuge** from Beckman Instruments includes refrigerated and versatile general-purpose tabletop centrifuges (*Reader*

Service No. 120). The compact centrifuge accommodates 1.5- to 100-ml tubes, 180-ml bottles, and microtitre and deep-well plates. It is fitted with a brushless induction drive that generates up to 17,530 g. Safety features of the GS-15 include diagnostics for overtemperature, imbalance and overspeed, a steel barrier ring around the chamber, and optional rotor bucket covers. Three types of rotors are available: the 45-degree fixed angle rotor accommodates 24 1.5-ml micro-centrifuge tubes, the 35-degree fixed angle rotor spins ten 10-ml tubes, and the 30-degree fixed angle rotor accommodates six 30-ml tubes. The microtitre rotor accommodates up to six microtitre plates per run, and the swinging bucket rotor holds four 180-ml bottles. The GS-15 reaches speeds up to 14,000 r.p.m. with fixed angle rotors and, using the S4180 swinging bucket rotor, can use modular adapters for common labware and bucket covers for containment.

The Howe-Sigma range of laboratory **centrifuges** offers up to 10 models for various applications in molecular biology. These include the Sigma 3K30 centrifuge, which combines a small benchtop framework with the performance of a high-speed centrifuge (*Reader Service No. 121*). As a result of its 64,000-g performance, V. A. Howe says that the 3K30 is useful for density gradient work for the isolation of materials such as lysosomes, mitochondria, peroxisomes, thylakoids and chloroplasts. The centrifuge is capable of accommodating up to 12 fixed-angle rotors, including an 8 × 50-ml rotor for Sorvall tubes, as well as a swing-out rotor and drum rotor. With the added facility of custom-making rotors, the centrifuge can always be upgraded to meet new needs. The frequency-controlled induction motor is designed to ensure smooth, quiet and vibration-free operation, in addition to a low maintenance requirement. Microprocessor control allows preselection of up to 20 acceleration/deceleration curves, direct input of the radial centrifugal force, and storage of up to 100 run programmes.

A new colour brochure from Du Pont describes the features and benefits that allow the Sorvall Super T 21 **centrifuge** to offer the capacity, performance and g-force capabilities usually found only in larger floor models (*Reader Service No. 122*). In one benchtop centrifuge, the instrument provides some of the features and capabilities of multiple instruments — a refrigerated microfuge for processing DNA samples, a lowspeed tabletop for performing cell separations and a floor model Superspeed for general purpose subcellular applications. The new Sorvall Super T 21 uses a chlorofluorocarbon replacement as a refrigerant. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.



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